University of Alberta

The Use of Lysozyme-HCl and Nisin to Control the Causal Agent of Chalkbrood Disease (*Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir) in Honey Bees (*Apis mellifera* L.)

by

Amanda Louise Van Haga

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

Biological Sciences

Fall 2010
Edmonton, Alberta

Examining Committee

B. Andrew Keddie, Biological Sciences

Stephen F. Pernal, Agriculture and Agri-Food Canada

Randolph S. Currah, Biological Sciences

Lloyd M. Dosdall, Agriculture, Food and Nutritional Science

Dedication

To my parents whose love and support has known no bounds. I am so grateful that you have taken all my endeavours seriously and we have seen this one to the end.

Abstract

Chalkbrood, caused by *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltor, is a cosmopolitan fungal disease of honey bee larvae (*Apis mellifera* L.) for which there is no chemotherapeutic control. Using *in vitro* larval rearing methods, lysozyme-HCl, a food-grade antimicrobial extracted from hen egg albumen, was found to suppress chalkbrood at levels of 0.75-1.5% (µg/mL) of larval diet. In field trials, lysozyme-HCl did not affect adult bee survival or brood production and did effectively suppress the development of chalkbrood disease. Daily chalkbrood mummy production decreased by a factor of 10 in colonies treated with three treatments of 6000 mg of lysozyme-HCl when compared with infected, untreated controls and reduced disease symptoms to levels observed in uninfected colonies. Honey production was also found to be significantly negatively correlated with increased disease severity. Lysozyme-HCl is a promising safe therapeutic agent for the control of chalkbrood in honey bee colonies.

Acknowledgements

I am grateful to Dr. Andrew Keddie for his support and guidance. His ability to think big and draw connections from the unlikeliest of places has been an inspiration. I also thank the members of my committee, Drs. Lloyd Dosdall and Randall Currah for their suggestions, guidance and constructive review of this thesis.

It is with deepest gratitude I thank Dr. Stephen Pernal. His sound advice, encouragement, and continuous support have made this thesis possible. As a mentor, he has been generous with his time and counsel and I have learned so much from him already. I have very much appreciated his thoroughness and attention to detail. I am also indebted to Adony Melathopoulos, whose patience, friendship and vast knowledge of all things bee related and beyond has made this work a joy. Both Steve and Adony have shown a passion and enthusiasm for research that is contagious and I look forward to working with them in the future.

Funding for this research has been provided by Canadian Bee Research Fund, Bee Maid Honey, Alberta Beekeepers' Commission, Neova Technologies and the Matching Investments Initiative (AAFC). I also thank the University of Alberta for the award of two travel grants: J Gordin Kaplan Graduate Student Award and Biological Sciences Travel Award.

Thank you to all of the summer students and support staff at Beaverlodge Research Farm, especially Peter Mills, Sterling Smith, Robert Albright, Corey White, Devon Coupland, Diana Munro, Laith Semonian, Robert Annett, Brandon Smith, Kelly Camsell, Merv Hegland, Mary Ann Oliver and "Queen Bee" Wendy Walter.

Lastly a big thank you to all the friends I have made while here at the U of A. I will greatly miss having lunches in the entomology lounge and constantly interrupting Jason, Marla, Lisa, Julian, Boyd, Bryan, Emily, Ben, Adam, Megan and Caroline while hard at work. Also, thanks to Helen who was always ready to listen over a glass of wine. Thank you for all of your support.

Table of Contents

Chapter 1: Literature Review 1

General Introduction 1

Study Organism: Honey Bee (*Apis mellifera* L.) 3

The Honey Bee Colony 3

Brood Rearing and Larval Development 5

Impacts of Modern Beekeeping on Disease 6

Study Organism: Chalkbrood (*Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir) 7

Description and Life Cycle 7

Transmission and Distribution 10

Factors Affecting Disease Expression in Colonies 12

Control of Chalkbrood Disease 15

Treatment Candidates: Lysozyme-HCl and Nisin 16

Thesis Goals 21

Literature Cited 22

Chapter 2: The Acute and Chronic Oral Toxicity of Lysozyme-HCl and Nisin to Adult Worker Bees and the *in Vitro* Efficacy of Lysozyme-HCl for the Treatment of Chalkbrood Disease 33

Introduction 33

Methods 37

Acute Oral Toxicity 37

Chronic Oral Toxicity 39

***In vitro* Larval Rearing Assay** 40

Results 42

Acute Oral Toxicity 42

Chronic Oral Toxicity 42

***In vitro* Larval Rearing Assay** 43

Discussion 44

Literature Cited 56

Chapter 3: The Effects of Chalkbrood Disease on Honey Bee Colonies in Northern Alberta and the Efficacy of Lysozyme-HCl in the Suppression of Chalkbrood Disease in the Field 61

Introduction 61

Methods 64

Colony Establishment and Management 64

Colony Inoculation 65

Colony Treatment 66

Colony Assessments 67

Statistical Analysis 70

Results 72

Disease Severity and Adult Bee Mortality 72

Colony Strength 76

Honey Production 76

Stability and Persistence of Lysozyme-HCl 77

Presence of Spores 77

Winter Survival 78

Discussion 78

Literature Cited 105

Chapter 4: Synthesis and Future Research 110

Research Summary 110

Applications and Recommendations 117

Future Research 119

Literature Cited 123

Appendix 1: Chapter 3 Statistical Tests and Results 128

List of Tables

Table 2.1. Acute oral toxicity of OTC, lysozyme-HCl (Inovapure™ 300), nisin (Novasin™) and dimethoate: LD_{50} for worker honey bees aged 7-9 d prior to exposure 54

Table 2.2. The chronic oral toxicity of lysozyme-HCl (Inovapure™ 300) to adult worker honey bees at 7, 14, and 21 d..... 55

Table 2.3. The chronic oral toxicity of nisin (Novasin™) to adult worker honey bees at 7, 14, and 21 d..... 55

Table 3.1. Percentage of samples (adult bee digestive tracts and larvae) from each treatment group collected weekly that were identified as chalkbrood positive..... 104

List of Figures

Figure 2.1. Cages (7.6 x 11.4 x 5.1 cm outside dimensions) containing 100 newly- emerged worker honey bees used in the chronic oral toxicity trials..... 49

Figure 2.2. Single frame queen excluders 49

Figure 2.3. Various stages of the life cycle of honey bees reared in vitro 50

Figure 2.4. Fourth-instar larvae showing signs of fungal growth at the posterior end..... 51

Figure 2.5. Signs of chalkbrood 51

Figure 2.6. Protective effects of 0, 0.75, 1.5 and 3% lysozyme-HCl (inovapure^{TM} 300) against larvae inoculated with 1x10^{8} spores A. apis/mL larval diet 52

Figure 2.7. Percentage of dead larvae pre and post defecation killed by chalkbrood..... 53

Figure 3.1. Dead bee traps (modified from Illies et al. 2002) attached to front of experimental colonies shown from the front and the side 87

Figure 3.2. Removable sheets facilitated the counting of chalkbrood mummies removed from the comb by bees..... 87

Figure 3.3. Colony populations assessed using a Plexiglas™ grid with 2.5 x 2.5 cm squares..... 88

List of Figures (continued)

Figure 3.4. The mean number of mummies (total, black, white) collected daily from traps and bottom boards for each treatment group from 14 May – 28 August 2007 89

Figure 3.5. The mean number of visible chalkbrood mummies (black and white) in brood frames counted weekly for each treatment group from 14 May – 28 August 2007 90

Figure 3.6. The mean numerical difference in number of black mummies compared with white mummies collected daily from traps and bottom boards or counted weekly in brood frames 91

Figure 3.7. 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 – 28 August 2007 92

Figure 3.8. Mean number of chalkbrood mummies collected daily from traps and bottom boards for each treatment group from 14 May – 28 August 2007 93

Figure 3.9. The weekly cumulative number of chalkbrood mummies collected daily from traps and bottom boards for individual colonies within each treatment group 94

Figure 3.10. The mean number of dead adult bees collected daily from traps and bottom boards compared among treatment groups (14 May – 28 August 2007) 95

List of Figures (continued)

Figure 3.11. Mean number of adult bees per colony for each treatment group estimated every two weeks until 10 July 2007 96

Figure 3.12. Mean number of sealed brood cells per colony for each treatment group estimated every two weeks until 10 July 2007 97

Figure 3.13. Mean number of unsealed brood cells per colony for each treatment group estimated every two weeks until 10 July 2007 98

Figure 3.14. Mean honey production (kg) per colony compared among treatment groups from 14 May – 28 August 2007 99

Figure 3.15. The relationship between total mummies collected daily from traps and bottom boards and honey yield (kg)..... 100

Figure 3.16. The mean amount (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 101

Figure 3.17. The average number of adult bees, sealed brood cells, and unsealed brood cells counted on 14 May 2008 for each treatment group ... 102

Figure 3.18. The average number of visible chalkbrood mummies (black and white) in brood frames counted on 14 May 2008 for each treatment group 103

Figure 4.1. The disease pyramid for honey bees and chalkbrood 119

Chapter 1: Literature Review

General Introduction

The recent dramatic losses of honey bee colonies in Canada and around the world have focused public attention on the health status of the honey bee *Apis mellifera* L. (Currie et al. 2010; Neumann and Carreck 2010) but the study of bee health dates as far back as 4th century BC when Aristotle (384-322 B.C.) described a bee disease which "is indicated in a lassitude on the part of the bees and in malodorousness of the hive" (Historia animalium IX.40.626b). Written records of treatments of bee diseases through chemical means are almost as ancient; Virgil (70-19 B.C.) recommends a number of treatments for honey bees "weakened with wretched disease" ranging from burning fragrant resin to pounded oak-apples with dry rose petals or dried grapes with thyme (Georgics IV verses 251-280). Today, there are over 30 identified pests and pathogens of honey bees worldwide (Morse and Flottum 1997; Ellis and Munn 2005) and thousands of studies of assessing the efficacy of various chemotherapeutic treatments.

Originating in Africa (Whitfield et al. 2006), honey bees were exported by man all over the world and with few exceptions their pests and pathogens have accompanied them (Ellis and Munn 2005; vanEngelsdorp and Meixner 2010). Introduced to North America in the early 1600s by settlers to produce honey and wax (Crane 1999), honey bees have become increasingly important for crop

pollination (Klein et al. 2007) and approximately 80% of all insect pollination is attributed to honey bees (Pimentel et al. 1997). Since 1961 North American crops requiring managed pollinators such as honey bees have increased while stocks of honey bees have declined (Aizen et al. 2008). In Canada, the value of honey bee pollination alone is estimated at $2.3 billion annually (S. Pernal, AAFC, personal communication).

Chalkbrood (*Ascosphaera* spp.) is a fungal disease of not only honey bee larvae but other important commercial pollinators such as the alfalfa leafcutting bee *Megachile rotundata* F. and the blue orchard bee *Osmia lignaria propinqua* Cresson (Gilliam and Vandenberg 1997). Found worldwide, chalkbrood disease is rarely lethal to honey bee colonies but can reduce colony foraging capacity up to 49% (Heath 1982a) impacting both pollination efficacy and honey production. Although a wide range of chemicals have been evaluated for the control of chalkbrood, none has been successfully adopted for use in North America (Aronstein and Murray 2010). 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 the residues of drugs 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; Martel et al. 2006). Interest in the study and use of natural compounds as an alternative to antibiotics to control

honey bee diseases has increased in recent years especially as concerns over antibiotic resistance grow (Davis and Ward 2003).

Study Organism: Honey Bee (*Apis mellifera* L.)

Reviewed extensively by Winston (1987), the honey bee belongs to the diverse hymenopteran family Apidae which is characterized by the presence of a pollen basket or corbicula on the hind tibia and whose members all exhibit some degree of social behaviour. The honey bee is eusocial and forms colonies comprising three physically distinct castes: queen, worker and drone. Though containing thousands of individuals, the honey bee colony can be considered a superorganism where individual actions are determined by colony needs (Seeley 1989) and reproductive success is defined in terms of the colony and not the individual (Moritz and Fuchs 1998). In temperate regions, colonies reproduce by swarming (colony fission) in the early spring and summer to ensure survival in the winter months, which is dependent on the stores of pollen and honey collected during summer (Winston 1992).

The Honey Bee Colony

The queen is the only reproductively functional female in the colony, laying upwards of 2000 eggs per day during peak egg production (Bodenheimer 1937). Honey bees are haplodiploid and the queen lays both fertilized eggs which develop into the female worker bee caste and haploid eggs which develop into reproductively functional males or drones. Drones comprise 5-10% of the colony

population in the spring and summer and their sole task is to mate with available virgin queens (Winston 1987; Boes 2010). Honey bee queens are polyandrous and mate on average with 12 males during their nuptial flight (Tarpy and Nielsen 2002). Higher mating frequency by honey bee queens has a number of beneficial effects on the colony as a whole (Tarpy and Seeley 2006). Increased genetic diversity in the colony increases disease resistance (Tarpy and Seeley 2006), colony productivity and fitness (Mattila and Seeley 2007) as well as enhancing the ability of the colony to recover from disturbances (Oldroyd and Fewell 2007).

Worker bees comprise the largest caste and perform all other colony-related tasks including: cell cleaning, brood tending, queen tending, receiving nectar, handling pollen, comb building, cleaning debris from the hive, ventilation, guard duty and foraging (Winston 1987). Within the worker caste is a temporal division of labour and although overlap and multi-tasking by individual bees is common, specific tasks tend to be performed by workers of a certain age. Task transition is gradual and occurs as a worker bee ages; cell cleaning and brood rearing are among the first tasks performed by worker bees upon emergence. However, foraging, a high risk activity, is typically the last task performed in a worker bee's life (Winston 1987). Worker bees demonstrate great flexibility in performing age-related tasks important to colony fitness throughout their lifetime (Winston 1992). In the absence of an older aged cohort, younger bees will become precocious foragers and 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). During the summer months, the lifespan of worker bees ranges between 25 and 35 days (Amdam and Omholt 2002)

Brood Rearing and Larval Development

A major component of brood tending activities is the nursing or feeding of larvae (Winston 1992). Individual cells are inspected thousands of times by numerous worker bees and when it is determined that larvae require feeding, a droplet of brood food is placed near the mouth of the larva. Brood food is composed of worker bee hypopharyngeal and mandibular gland secretions; as larvae age, their feeding requirements change and pollen and honey are incorporated into their diet (Haydak 1970; Winston 1987). Constantly feeding, honey bee larvae undergo five molts. Larval development time varies between castes and ranges between 5-6 days for worker bee larvae. Prior to pupation, the cells are sealed with wax cappings by adult worker bees and the enclosed larvae complete feeding and defecate. Stretching out in the cell with their heads oriented toward the capping, larvae spin cocoons and pupate. The pupal stage lasts 8-9 days after which the newly-formed worker adult bees chew their way out of the cell, eat and begin to work (Winston 1987). Total development time for worker bees, from the time of egg laying by the queen to emergence as an adult, ranges between 21 and 24 days (Winston 1987).

Both the colony environment and the adult worker bees can affect the development of the larvae. Adult nurse bees are important vectors and transmit

viral and spore-forming pathogens to the larvae during feeding which could lead to death or delayed development (Peng et al. 1992). In addition, any treatments applied to the colony or chemicals from pesticides or herbicides contaminating the incoming food resources may also be passed to larvae though the brood food (Davis and Shuel 1988; Peng et al. 1992). Reduced adult worker bee populations or decreased food resources can have a significant impact on both the quantity and quality of the brood and may reduce the health and productivity of the next generation of adult worker bees (Winston 1987).

Impacts of Modern Beekeeping on Disease

The earliest known record of domestically keeping honey bees for honey and wax dates as far back as 2400 B.C. in Egypt (Crane 1999), but modern beekeeping as we know it today began in 1851 when Langstroth revolutionized beekeeping with the invention of the moveable frame hive (Crane 1992). Although moveable frame hives have facilitated identification of pests and pathogens in the colony by allowing easy inspection of the brood nest (Crane 1999), they have also impacted the spread of bee diseases in other ways. Current management practices like swarm prevention, combining weak colonies together or adding frames of bees and brood to increase colony strength have resulted in higher densities of bees within in a single colony. The interchange of equipment and bees between colonies, the increased density of bees in a single colony and the practice of keeping many colonies together in one location can increase horizontal transmission of pathogens between individual bees and colonies (Fries and

Camazine 2001; James 2008). Additionally, colony management to reduce swarming or production of daughter colonies decreases vertical transmission of pathogens, and may lead to increased virulence (Fries and Camazine 2001).

Study Organism: Chalkbrood (*Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir)

Description and Life Cycle

Comprehensively reviewed by Heath (1982a, b, 1985), Hornitzky (2001) and Aronstein and Murray (2010), chalkbrood is a fungal disease of honey bee larvae caused by the heterothallic fungus *Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir (Spiltoir and Olive 1955). The name is derived from the mummified chalky cadavers that are produced when larvae are infected with the fungus. The first description of chalkbrood was in 1913 (Maassen 1913 as cited in Spiltoir and Olive 1955) when *A. apis* was originally identified as *Pericystis apis* (Maassen 1916 as cited in Spiltoir and Olive 1955). It was reclassified in 1955 (Spiltoir and Olive 1955) and the current accepted taxonomic lineage is Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Onygenales; Ascosphaeraceae; *Ascosphaera apis* (Lumbsch and Huhndorf 2007). Different species of *Ascosphaera* have been shown to cause chalkbrood in other bee species such as the alfalfa leafcutter bee *Megachile rotundata* F. (caused by *Ascosphaera aggregata* Skou) (Vandenberg and Stephen 1982) and the orchard mason bee *Osmia lignaria propinqua* Cresson (caused by *Ascosphaera torchioi* Youssef and

McManus) (Torchio 1992) however *A. apis* is the sole etiological agent of chalkbrood in honey bees (Puerta et al. 1999).

A. apis is heterothallic and produces spores only when mycelia of opposite mating types come into contact (Spiltoir 1955). Mating types are morphologically identical and are indistinguishable from each other when cultured separately (Spiltoir 1955; Heath 1982b). When cultured, the mycelium is dense and white and the hyphae grow on and below the surface as well as aerially (Spiltoir 1955; Anderson and Gibson 1998). The hyphae (2.5-8 µm d) are straight, smooth walled, dichotomously branched, and septate (Spiltoir and Olive 1955; Skou 1988; Anderson and Gibson 1998). Within the fruiting bodies or spore cysts (36-131 µm d) that are formed, are spore balls (6-19 µm d) which contain many tightly packed spores (Anderson and Gibson 1998; Aronstein and Murray 2010). The spores of *A. apis* are hyaline, ellipsoidal to reniform in shape (Heath 1982b) and range in size from 2.7-3.5 x 1.4-1.8 µm (Aronstein and Murray 2010). Extremely hardy, spores can remain infective for up to 15 years when stored at ambient temperatures (Toumanoff 1951 as cited in Jensen et al. 2009a). It has also been shown that storage for one year at -80°C does not decrease spore viability (Jensen et al. 2009a).

The spores of *A. apis* circulating within the colony are transmitted to honey bee larvae via nurse bees during feeding. Once ingested, they germinate in the lumen of the midgut, particularly at the posterior region (Bamford and Heath 1989;

Gilliam and Vandenberg 1997; Aronstein and Murray 2010). Germination is most likely activated by CO_2 which is produced by the larvae during respiration (Heath and Gaze 1987). The mycelium, however, cannot grow anaerobically (Heath 1982b). Although larvae between one- and four-days old are susceptible to infection (Aronstein and Murray 2010), larvae three- to four-days old are more susceptible than younger larvae. This may be because the mycelia are not subjected as long to the anaerobic conditions of the digestive tract in older aged larvae (Bailey 1968). Larval chilling, suggested to increase the amount of oxygen in the tissues, also facilitates mycelial growth and has been shown to increase incidence of chalkbrood infection and mummification (Bailey 1967; Flores et al. 1996).

The vegetative growth of *A. apis* breaches the peritrophic membrane lining the larval midgut through enzymatic degradation and mechanical pressure exerted by the growing hyphae (Bamford and Heath 1989; Alonso et al. 1993). The hyphae proliferate in the hemocoel and grow through the cuticle spreading from the posterior end until the entire larva is covered in fungal growth (Chorbiński 2004; Aronstein and Murray 2010). The characteristic hard chalk-like mummies result from the drying of the larval mycelial mass in the brood cell (Heath 1982a). The presence of fruiting bodies that turn brown to black when mature (Anderson and Gibson 1998) give mummies a grey-black appearance, with each mummy containing between 10^8 and 10^9 spores (Nelson and Gochnauer 1982). It is commonly thought that in the absence of fruiting bodies, chalkbrood mummies

are white (Bailey 1968) and it has been suggested that a single strain may out-compete other strains of *A. apis*, remain unmated, and not produce fruiting bodies (Christensen and Gilliam 1983). However, the ability of a single strain to inhibit the growth of all other strains does not occur when white mummies are cultured in the laboratory, and it has been hypothesized that younger mummies are white and have not had time or proper conditions for the ascospores to develop and mature (Aronstein and Murray 2010).

Chalkbrood mummies are removed from brood cells by worker bees and deposited outside the hive (Heath 1982a). During the cleaning of the diseased cells, spores can be transferred to the body hairs or ingested and can be spread through the colony via oral trophallaxis or contact between adult bees. Spores accumulate in the pollen and honey stored in the hive (Puerta et al. 1999) and have also been detected in the wax foundation that workers use to build comb for brood rearing or storage of honey and pollen (Flores et al. 2005).

Transmission and Distribution

Transmission of chalkbrood within and between apiaries can occur when honey bees rob or drift among other colonies. Additionally, common forage sites and water sources can act a reservoir for spores (Heath 1982a). It is possible that wild bee species play a role in the spread of chalkbrood in honey bees as *A. apis* has been detected in various leafcutter bees, soil-nesting bees, mason bees and alkali bees (Heath 1985; Gilliam and Vandenberg 1997). Beekeepers themselves are

important vectors and can spread spores through their operations through use of contaminated equipment and bee products (Heath 1982a). The ease with which the resilient *A. apis* spores are spread and transmitted is reflected in the rapid spread of chalkbrood worldwide (Ellis and Munn 2005; Castagnino et al. 2006; Rundassa 2006). A commonly recognized disease in Europe in the early half of the 20th century, chalkbrood was not reported outside of Europe until the 1950s (Aronstein and Murray 2010) and is now found on all continents with honey bees. Chalkbrood disease in honey bees was not reported in North America until 1968 (Baker and Torchio 1968) and the first report in Canada was in 1971 (Gochnauer et al. 1972). By 1985, chalkbrood had spread throughout North American (Heath 1985) and is attributed primarily to the extensive movement of bees and equipment by commercial beekeepers prior to that time (Aronstein and Murray 2010).

Studies provide evidence that strains of *A. apis* show varying degrees of virulence and differences in virulence have been reported to be as high as 20-fold (Gliński 1982). Jākobsons (2005) found that strains isolated from Thailand were more virulent than German strains when tested against larvae from Israeli colonies indicating that virulence could vary between geographical regions. The factors that influence virulence are not yet identified but it is theorized it is an array of characteristics and not a single phenotypic property (Theantana and Chantawannakul 2008). The increase in chalkbrood incidence in recent years in North America may be the result of newly-introduced *A. apis* strains that are

either novel to the established bee populations or more virulent than the existing strains.

Factors Affecting Disease Expression in Colonies

Outbreaks of chalkbrood disease are unpredictable and development of chalkbrood disease in the colony is influenced by a number of factors. *Ascosphaera apis* spores have been detected in colonies with no visible symptoms (chalkbrood mummies) (Gilliam 1986) and larvae can ingest spores without developing the disease (Heath 1982a). Chalkbrood is considered a factorial disease requiring both the presence of spores in the larval gut and environmental stress before it is expressed (Flores et al. 2004).

An important predisposing condition that can significantly increase chalkbrood infection is chilling of the brood (Heath 1982a). Normal hive temperatures are 33-35°C (Winston 1987) and even small drops in temperature of 3-5°C can significantly increase mummification (Bailey 1967). Larvae are most susceptible to chilling immediately following capping of the brood cell and short periods of cooling (6 hours) can cause significant increases in infection (Bailey 1967). Outbreaks of chalkbrood are more commonly reported in the spring when temperatures are cooler and the colony is rapidly increasing in size (Heath 1982a). The rapid buildup of the colony in the spring can decrease the ratio of adult bees to brood resulting in uneven hive temperatures or reduced brood care (Heath 1982a; Koenig et al.1987). Drone larvae are especially at a higher risk of

infection as they are found mainly on the periphery of the brood nest where temperature fluctuations are more likely (Anderson 1934). Nevertheless Gilliam et al. (1978) reported high levels of year-round chalkbrood infection in colonies in Arizona where average monthly temperatures were 29°C and risk of larval chilling was low, suggesting other factors might also be as important.

High levels of humidity in the colony have also been implicated a major factor in disease induction (Heath 1982a), based on the observed frequencies of chalkbrood in damp regions or during times associated with high colony humidity and poor ventilation (Flores et al. 1996; Gilliam and Vandenberg 1997). Humid conditions do enhance fungal growth in species other than *A. apis* in the colony but experimentally, mummification of larvae increased only when increased relative humidity (68% to 87%) was combined with brood chilling (Flores et al. 1996).

Honey bee larvae may also be more susceptible to chalkbrood infection when the colony is under stress. Lack of food or exposure to other pests and pathogens can have an indirect effect on chalkbrood development by reducing the quality of brood care or compromising the immune systems of individuals (Heath 1982a; Flores et al. 2005; Aronstein and Murray 2010). Examples of other colony stress that can aggravate chalkbrood disease include heavy manipulation of frames within the hive that can interfere with larval care leading to nutritional deficiencies and chilling of the larvae (Befus-Nogel et al. 1992) or colony relocation which can heavily disrupt foraging activities leading to food shortages

(Jay 1986). Other diseases or pathogens can severely decrease colony populations (Deans 1940) and affect individual bee immunity. Mehr et al. (1976) postulated that sacbrood, a viral disease of larvae, decreased larval resistance to chalkbrood infection. Colonies that are infested with the parasitic mite *Varroa destructor* Anderson and Truemann or tested positive for *Nosema apis* Zander spores had higher levels of chalkbrood than uninfested colonies (Gliński 1991; Aydin et al. 2006).

In combination with environmental factors, natural variation in susceptibility to chalkbrood infection among colonies has been documented. A major component of chalkbrood resistance in honey bee colonies is hygienic behaviour (Spivak and Gilliam 1993). The detection and removal of diseased larvae by adult worker bees or hygienic behaviour is a heritable colony-level trait important in disease resistance (Rothenbuhler 1964) and studies have shown that colonies that exhibit this behaviour are more resistant to chalkbrood (Gilliam et al. 1983; Spivak and Reuter 1998). Hygienic behaviour 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 odours is higher in honey bees bred for hygienic behaviour than in bees from non-hygienic lines (Gramacho and Spivak 2003; Masterman et al. 2001). Recently it was demonstrated that the volatile compound phenethyl acetate, isolated from larvae infected with *A. apis* that had not sporulated, was

capable of inducing hygienic behaviour in the field (Swanson et al. 2009). Additionally, evidence suggests that there are physiological mechanisms such as naturally occurring anti-mycotics isolated from nurse bee guts and stored pollen antagonistic to *A. apis*, which also contribute to chalkbrood resistance (Spivak and Gilliam 1993).

Differential resistance to chalkbrood infection in larvae of different genotypes has also been demonstrated. In twenty-two different subfamilies or paternal lines within a single colony, chalkbrood infection ranged from 17.6% to 100% (Invernizzi et al. 2009). Increased genetic diversity (queen promiscuity) within a colony has also been shown to reduce intensity of chalkbrood infection and other diseases in the field (Tarpy 2003). In laboratory experiments, larvae reared outside of colony environments inoculated with *A. apis* spores varied in susceptibility to chalkbrood infection within and between different subspecies of honey bees (Jensen et al. 2009b). Although variation in larval tolerance to *A. apis* exists, heritability has not yet been established.

Control of Chalkbrood Disease

The lack of successful chemotherapeutic agents for the treatment of chalkbrood disease has led to implementation of various management practices to control incidence and spread of chalkbrood within beekeeping operations (Heath 1982a; Aronstein and Murray 2010). Hive equipment disinfection practices including scorching or bleaching of equipment reduces the number of *A. apis* spores as well

as other honey bee pathogens (Shimanuki and Knox 1997). Comb can also be sterilized by irradiation (Melathopoulos et al. 2004) and comb replacement is an effective way to reduce chalkbrood disease in operations (Nelson and Gochnauer 1982). Often in severely infected colonies, honey bee queens are removed and replaced with queens from a chalkbrood-resistant stock line (Gilliam and Vandenberg 1997). Weak colonies are strengthened with the addition of either more brood or bees and to prevent nutritional stress colonies are supplemented with pollen and/or syrup (Heath 1982a). Additionally, it is recommended that colonies are not placed in shady low-lying areas where there is a higher risk of chilling or dampness (Aronstein and Murray 2010).

Treatment Candidates: Lysozyme-HCl and Nisin

Described in 1922 by Alexander Fleming, lysozyme was first isolated from his own nasal mucus that was found to be bacteriolytic and was further identified in a number of human secretions (Fleming 1922). Lysozymes are a family of enzymes found in a variety of sources in nature including arthropods and plants (Johnson and Larson 2005). Endogenous lysozymes play an important role in cellular immune responses in arthropods (Gliñski and Jarosz 2001) and have been detected in the salivary glands of worker honey bees (Bodnarchuk et al. 2003) and in the haemolymph of both the larvae and adults (Aronstein and Saldivar 2005). Part of the inducible immune defenses of insects, lysozymes are upregulated upon infection (Gillespie et al. 1997) and in honey bee larvae, levels of lysozyme increase 500 times the normal levels when infected artificially with bacteria

(Gliñski and Jarosz 2001). Gliñski and Buczek (2003) stated that neither lysozyme nor other inducible antimicrobial peptides found in the haemolymph of honey bees have been found to protect or act against fungal infections but Aronstein et al. (2010) found that expression of Honey bee C-type lysozyme-1 significantly increased 24 h post infection in one day old larvae inoculated with *A. apis* spores. Nevertheless, phagocytosis and encapsulation remain the most effective immune response of honey bees to fungal invaders (Gliñski and Buczek 2003; Aronstein et al. 2010).

Hen egg albumen is the primary source of commercial grade preparations of lysozyme. Through extraction methods using NaCl, lysozyme is modified as a hydrochloride salt that is freely soluble in water and retains its original properties (Johnson and Larson 2005). Lysozyme-HCl is used extensively in pharmaceuticals and food preservation as it as an extremely stable, non-toxic antimicrobial. Lysozyme-HCl remains stable and active over a wide range of temperatures (up to 100°C) and pH, but is most active within a pH range of 3.5-7.0 (Proctor and Cunningham 1988). Lysozyme is a reversible dimer between the pH of 5.0 and 9.0 (Lesnierowski and Kijowski 2007) and in addition to bacteriolytic activity; the dimer has therapeutic, anti-viral and anti-inflammatory properties that the monomer does not (Cegielska-Radziejewska et al. 2008). Lysozyme-HCl has been granted GRAS (generally regarded as safe) status by the American Food and Drug Administration and accepted for use in food processing by the Joint FAO/WHO Expert Committee on Food Additives (Johnson and

Larson 2005). Primarily, lysozyme-HCl is used to prevent spoilage in cheeses and wine by inhibiting the growth of Gram-positive bacteria but it has also been used as a preservative in a number of food products including seafood, meats, fruits and vegetables (Proctor and Cunningham 1988).

Gram-positive bacteria with cell walls rich in peptidoglycan are particularly susceptible to lysozyme-HCl. The main mode of action of lysozyme is the hydrolysis of the ß(1-4) linkages in the polymers that lead to cell lysis (Strominger and Tipper 1974). Chitin, a component of most fungal cells walls (Griffin 1994), also contains ß(1-4) glycosidic bonds that can be hydrolyzed by lysozyme-HCl (Berger and Weiser 1957). Although not as common because of the low peptidoglycan content in the cell wall and the protective outer layer composed of lipids, lysozyme-HCl is also active against some Gram-negative bacteria (Johnson and Larson 2005). Nevertheless, the antimicrobial activity of lysozyme-HCl is not solely dependent on its enzymatic activity and studies have shown that even when denatured by heat, lysozymes were still capable of suppressing growth of Gram-negative bacteria (Ibrahim et al. 1996). Embedded in the structure of lysozyme-HCl are amphipathic helical regions that are capable of cell membrane disruption by distorting lipid-lipid interactions and increasing membrane permeability (Ibrahim et al. 1996; Düring et al. 1999). In addition to cell membrane disruption, lysozymes are highly cationic and may be capable of inducing autolysis in bacteria (Ginsburg and Koren 2008).

The effects of lysozyme on fungi are not as well studied as its effects on bacteria. Research into the fungistatic effects of lysozyme has focused on the medically important unicellular yeast *Candida albicans* (C.P. Robin) Berkhout. However, *Aspergillus fumigatus* Fresen, *Rhizopus oryzae* Went & Prins. Geerl, *Histoplasma capsulatum* Darling and *Paracoccidioides brasiliensis* (Splend.) F.P. Almeida are also susceptible to lysozyme (Lopera et al. 2008). Investigations on *P. brasiliensis*, a member of the Ascomycota order Onygenales, shows that lysozyme plays a dual role, causing ultra-structural changes in the conidia (asexual spores) and inhibiting conidium to yeast cell transitions and yeast budding (Lopera et al. 2008).

The combination of lysozyme with compounds such as other naturally occurring antimicrobials like nisin or lactoferrin, chelators like EDTA, or antibiotics and antimycotics like amphotericin B has been shown to enhance activity against selected microorganisms (Collins and Pappagianis 1974; Johnson and Larson 2005). Nisin, a broad-spectrum antimicrobial produced by *Lactococcus lactis* Lister during fermentation, is also used in food preservation and has GRAS status (Takala and Saris 2007). Like lysozyme, nisin inhibits growth of Gram-positive bacteria. It causes cell death by creating pores in cell membranes that make cells permeable to ionic components (Bruno et al. 1992). Further, it induces autolysis in cells, inhibits cell wall synthesis (Takala and Saris 2007) and prevents spore outgrowth (Abee and Delves-Broughton 2003). In laboratory experiments, nisin was shown to inhibit the growth of *Paenibacillus larvae* (sensu Genersch et al.

2006), the Gram-positive bacterium that causes American foulbrood, a serious disease of honey bee larvae (Chacana et al. 2003).

Studies on the synergistic effects of lysozyme/nisin combinations demonstrate enhanced activity against Gram-positive bacteria for both compounds that may be attributed to complementary modes of action (Proctor and Cunningham 1993; Chung and Hancock 2000). The use of a combination of compounds for treatment of diseases is preferable to single drug treatments as it reduces the amount of both drugs required and is considered the optimal strategy to prevent the development of microbial resistance through mutation or gene exchange (Bonhoeffer et al. 1997). Because natural antimicrobials like lysozyme are non-selective inhibitors and generally do not target specific cell sites like antibiotics, (Davidson and Harrison 2002) incidences of acquired resistance in natural antimicrobials are less prevalent than in antibiotics. Although not well studied, development of resistance over time to lysozyme and nisin by various bacteria has been reported in laboratory experiments (Litwack and Prasad 1962; Gravesen et al. 2002). Acquired resistance to lysozyme in Gram-positive bacteria results from modifications to the cell wall by O-acetyl or N-acetyl groups (Brumfitt et al. 1958; Masschalck and Michiels 2003) but resistance is often unstable and bacteria revert to susceptibility or "back-mutate" when lysozyme is removed from the growth medium (Brumfitt 1959; Russel 1991). There has, however, been evidence of stable acquired resistance to lysozyme by *Bacillus subtilis* in laboratory experiments (Özcengiz and Alaeddinoglu 1991). Consequently, the

possibility of resistance to lysozyme-HCl when used a therapeutic agent for honey bee larval diseases should not be ignored.

Thesis Goals

The aims of this thesis are to:

1. Evaluate the toxicity of lysozyme-HCl and nisin for to adult honey bees using oral toxicity cage studies,
2. If non-toxic, assess the efficacy of lysozyme-HCl and nisin against chalkbrood disease in honey bee larvae reared *in vitro*,
3. If efficacious *in vitro*, determine a therapeutic dose for chalkbrood control at the colony level and lastly,
4. Assess the impacts of chalkbrood disease on honey bee colonies.

Literature Cited

Abee, T., and J. Delves-Boughton. 2003. Bacteriocins - Nisin, pp. 146-178. In N. J. Russell & G. W. Gould (Eds.), Food preservatives. Kluwer Academic/Plenum Publishers, New York, N.Y.

Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., and A.M. Klein. 2008. Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. Curr. Biol. 18:1572-1575.

Alonso, J.M., Rey, J., Puerta, F., Hermoso de Mendoza, J., Hermoso de Mendoza, M., and J.M. Flores. 1993. Enzymatic equipment of *Ascosphaera apis* and the development of infection by this fungus in *Apis mellifera*. Apidologie 24:383-390.

Amdam, G.V. and S.W. Omholt. 2002. The regulatory anatomy of honeybee lifespan. J. Theor. Biol. 216: 209-228.

Anderson, D.L., and N.L. Gibson. 1998. New species and isolates of spore-cyst fungi (Plectomycetes: Ascosphaerales) from Australia. Aust. Syst. Bot. 11: 53-72.

Anderson, J. 1934. Brood disease in Scotland, pp.17-18. In E.J. Russell (ed), Rothamsted conferences, XVIII: Brood diseases of bees, being the report of a conference held at Rothamsted on May 19th, 1934 under the chairmanship of Sir E.J. Russell. Harpenden, Rothamsted Experimental Station, England.

Aristotle (384-350 B.C.). 1910. Historia animalium. Oxford University Press, Oxford, England.

Aronstein, K., and E. Saldivar. 2005. Characterization of a honeybee Toll related receptor gene Am18w and its potential involvement in antimicrobial immune defense. Apidologie 36: 3-14.

Aronstein, K.A., and K.D. Murray. 2010. Chalkbrood disease in honey bees. J. Invertebr. Pathol. 103: S20-S29.

Aronstein, K.A., Murray, K.D., and E. Saldivar. 2010. Transcriptional responses in honey bee larvae infected with chalkbrood fungus. BMC Genomics 11:391-403.

Aydin, L., Güleğen, E., Çakmak, İ., Girişgin, O., and H. Wells. 2006. Relation between nosema and chalkbrood diseases, and its implication for an apiary management model. Bull. Vet. Inst. Pulawy 50: 471-475.

Baker, G.M., and P.F. Torchio. 1968. New records of *Ascosphaera apis* from North America. Mycologia 60: 189–190.

Bailey, L. 1967. The effect of temperature on the pathogenicity of the fungus *Ascosphaera apis* for larvae of the honeybee, *Apis mellifera*, pp. 162–167. In P.A. Van der Laan [ed], Insect pathology and microbial control. North Holland Publishing Co., Amsterdam, Netherlands.

Bailey, L. 1968. Honey bee pathology. Annu. Rev. Entomol. 13:191-212.

Bamford, S., and L.A.F. Heath. 1989. The infection of *Apis mellifera* by *Ascosphaera apis*. J. Apic. Res. 28:30-35.

Befus-Nogel, J., Nelson, D.L., and P. Lefkovich. 1992. Observations on the effect of management procedures on chalkbrood levels in honey bee (*Apis mellifera* L; Hymenoptera: Apidae) colonies. Bee Sci. 2: 20-24.

Berger, L.R., and R.S. Weiser. 1957. The β-glucosaminidase activity of egg-white lysozyme. Biochim. Biophys. Acta. 26: 517-521.

Bodenheimer, F.S. 1937. Studies in animal population. II. Seasonal population trends of the honeybee. Quart. Rev. Biol. 12: 406-425.

Bodnarchuk, L., Nagornaya, I., and I. Levchenko. 2003. Factors that determine lysozyme level in the honey, pp.110-111. In Materialy z XL Naukowej Konferencji Pszczelarskiej, 11-12 March 2003, Pulawy, Poland.

Boes, K.E. 2010. Honeybee colony drone production and maintenance in accordance with environmental factors: an interplay of queen and worker decisions. Insectes Soc. 57: 1-9.

Bonhoeffer, S., Lipsitch, M., and B.R. Levin. 1997. Evaluating treatment protocols to prevent antibiotic resistance. Proc. Natl. Acad. Sci. USA 94: 12106-12111.

Brumfitt, W. 1959. The mechanism of development of resistance to lysozyme by some gram-positive bacteria and its results. Br. J. Exp. Pathol. 40: 441–451.

Brumfitt, W., Wardlaw, A.C., and J.T. Park. 1958. Development of lysozyme-resistance in *Micrococcus lysodiekticus* and its association with an increased O-acetyl content of the cell wall. Nature 181: 1783-1784.

Bruno, M.E.C., Kaiser, A., and T.J. Montville. 1992. Depletion of proton motive force by nisin in *Listeria monocytogenes* cells. Appl. Environ. Microbiol. 58: 2255-2259.

Castagnino, G.L.B., Funari, S.R.C., Blume, E., Arboitte, M.Z., and M.N. Weber. 2006. Chalk brood disease *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir in the honey bee *Apis mellifera* L. in Central Depression of Rio Grande do Sul. Ciênca Rural 36: 1909-1911.

Cegielska-Radziejewska, R., Leśnierowski, G., and J. Kijowski. 2008. Properties and application of egg white lysozyme and its modified preparations - a review. Pol. J. Food Nutr. Sci. 58: 5-10.

Chacana, P.A., Borracci, S.E., Vallejo, M., Palacio, M.A., and H.R. Terzolo. 2003. Sensibilidad de *Paenibacillus larvae* al antimicrobiano nisina [Sensitivity of *Paenibacillus larvae* to the antimicrobial nisin]. http://www.inta.gov.ar/balcarce/info/documentos/posters/22/paenabacillusChacanaimagen.htm.

Chorbiński, P. 2004. The development of the infection of *Apis mellifera* larvae by *Ascosphaera apis*. Electron. J. Pol. Agric. Univ. 7, #03 [online]. http://www.ejpau.media.pl/volume7/issue2/veterinary/art-03.html.

Christensen, M., and M. Gilliam. 1983. Notes on the *Ascosphaera* species inciting chalkbrood in honey bees. Apidologie 14: 291–297.

Chung, W., and R.E.W. Hancock. 2000. Action of lysozyme and nisin mixtures against lactic acid bacteria. Int. J. Food Microbiol. 60: 25-32.

Collins, M. S., and D. Pappagianis. 1974. Inhibition by lysozyme of growth of the spherule phase of *Coccidioides immitis* in vitro. Infect. Immun. 10: 616-623.

Crane, E. 1992. The world's beekeeping - past and present, pp. 1-20. In J.M. Grant (ed.), The hive and the honey bee. Dadant and Sons Inc., Hamilton, IL.

Crane, E. 1999. The world history of beekeeping and honey hunting. Routledge, New York, NY.

Currie, R.W., Pernal, S.F., and E. Guzmán-Novoa. 2010. Honey bee colony losses in Canada. J. Apic. Res. 49: 104-106.

Davidson, P.M. and M.A. Harrison. 2002. Resistance and adaptation to food antimicrobials, sanitizers, and other process controls. Food Technol. 56: 60-78.

Davis, A.R., and R.W. Shuel. 1988. Distribution of C-14-labeled carbofuran and dimethoate in royal jelly, queen larvae and nurse honeybees. Apidologie 19: 37-50.

Davis, C., and W. Ward. 2003. Control of chalkbrood disease with natural products. Publ. No. 03/107, Rural Industries Research and Development Corporation, Kingston ACT, Australia.

Deans, A.S.C. 1940. Chalk brood. Bee World. 21: 46.

Düring, K., Porsch, P., Mahn, A., Brinkmann, O., and W. Gieffers. 1999. The non-enzymatic microbicidal activity of lysozymes. FEBS Lett. 449: 93-100.

Ellis, J.D., and P.A. Munn. 2005. The worldwide health status of honey bees. Bee World 86: 88-101.

Fleming, A. 1922. On a remarkable bacteriolytic element found in tissues and secretions. Proc. R. Soc. Lond. B 93: 306-317.

Flores, J.M., Ruiz, J.A., Ruiz, J.M., Puerta, F., Busto, M., Padilla, F., and F. Campano. 1996. Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions. Apidologie 27: 185-192.

Flores, J.M., Gutierrez, I., and F. Puerta. 2004. Oxytetracycline as a predisposing condition for chalkbrood in honeybee. Vet. Microbiol. 103: 195-199.

Flores, J., Spivak, M., and I. Gutierrez. 2005. Spores of *Ascosphaera apis* contained in wax foundation can infect honeybee brood. Vet. Microbiol. 108: 141–144.

Fries, I., and S. Camazine. 2001. Implications of horizontal and vertical pathogen transmission for honey bee epidemiology. Apidologie 32:199–214.

Genersch, E., Forsgren, E., Pentikäinen, J., Ashiralieva, A., Rauch, S., Kilwinski, J., and I. Fries. 2006. Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. Int. J. Syst. Evol. Microbiol. 56: 501-511.

Gillespie, J.P., Kanost, M.R., and T. Trenczek. 1997. Biological mediators of insect immunity. Ann. Rev. Entomol. 42: 611-643.

Gilliam, M., Taber, S. III., and J.B. Rose. 1978. Chalkbrood disease of honey bees, *Apis mellifera* L.: a progress report. Apidologie 9: 75-89.

Gilliam, M., Taber, S. III., and G.V. Richardson. 1983. Hygienic behavior of honey bees in relation to chalkbrood disease. Apidologie 14: 29-39.

Gilliam, M. 1986. Infectivity and survival of the chalkbrood pathogen, *Ascosphaera apis*, in colonies of honey bees, *Apis mellifera*. Apidologie 17: 93-100.

Gilliam, M., and J.D. Vandenberg. 1997. Fungi, pp. 81–110. In R.A. Morse and K. Flottum (eds.), Honey bee pests, predators and diseases, 3rd ed. A.I. Root Company, Medina, OH.

Ginsburg, I., and E. Koren. 2008. Are cationic antimicrobial peptides also 'double-edged swords'? Expert Rev. Anti Infect. Ther. 6: 453-462.

Gliński, Z. 1982. Studies on pathogenicity of *Ascosphaera apis* for larvae of the honeybee *Apis mellifera* L. Part II. Relationships between biochemical types and virulence of *A. apis*. Ann. Univ. Mariae Curie-Sklodowska, Sect. DD 37: 69-77.

Gliński, Z. 1991. The effect of *Varroa jacobsoni* Oud. on the incidence and course of chalkbrood disease in *Apis mellifera* L. colonies. Rev. Agric. Entomol. 34: 79-97.

Gliński, Z., and K. Buczek. 2003. Response of the Apoidea to fungal infections. Apiacta 38: 183-189.

Gliński, Z., and J. Jarosz. 2001. Infection and immunity in the honey bee *Apis mellifera*. Apiacta 36: 12-24.

Gochnauer, T.A., Hughes, S.J., and J. Corner. 1972. Chalkbrood disease of honey bee larvae... a threat to Canadian beekeeping? Can. Agricult. 17: 36-37.

Gravesen, A., Jydegaard Axelsen, A.M., Mendes da Silva, J., Hansen, T.B., and S. Knøchel. 2002. Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. Appl. Environ. Microbiol. 68: 756-764.

Gramacho, K.P., and M. Spivak. 2003. Differences in olfactory sensitivity and behavioural responses among honey bees bred for hygienic behavior. Behav. Ecol. Sociobiol. 54: 472-479.

Griffin, D.H. 1994. Fungal physiology, 2nd ed. Wiley-Liss, New York, N.Y.

Haydak, M.H. 1970. Honey bee nutrition. Annu. Rev. Entomol. 15: 143-156.

Heath, L.A.F. 1982a. Development of chalk brood in a honey bee colony: a review. Bee World 63: 119-130.

Heath, L.A.F. 1982b. Chalk brood pathogens: a review. Bee World 63: 130-135.

Heath, L.A.F. 1985. Occurrence and distribution of chalkbrood disease of honey bees. Bee World 66: 9–15.

Heath, L.A.F., and B.M. Gaze. 1987. Carbon dioxide activation of spores of the chalkbrood fungus, *Ascosphaera apis*. J. Apic. Res. 26: 243-246.

Hornitzky, M. 2001. Literature review of chalkbrood–a fungal disease of honeybees. Publ. No. 01/150, Rural Industries Research and Development Corporation, Kingston ACT, Australia.

Ibrahim, H.R., Higashiguchi, S., Juneja, L.R., Kim, M., and T. Yamamoto. 1996. A structural phase of heat-denatured lysozyme with novel antimicrobial action. J. Agric. Food Chem. 44: 1416-1423.

Invernizzi, C., Peñagaricano, F., and I. H. Tomasco. 2009. Intracolonial genetic variability in honeybee larval resistance to the chalkbrood and American foulbrood parasites. Insect. Soc. 56: 233-240.

Jākobsons, B. 2005. Biological treatment of chalkbrood in honey bees. Ph.D. dissertation, Latvia University of Agriculture, Jelgava, Latvia.

James, R.R. 2008. The problem of disease when domesticating bees, pp124-141. In R.R James and T.L. Pitts-Sanger (eds.), Bee pollination in agricultural ecosystems. Oxford University Press, New York, NY.

Jay, S.C. 1986. Spatial management of honey bees on crops. Annu. Rev. Entomol. 31: 49-65.

Jensen, A.B., James, R.R., and J. Eilenberg. 2009a. Long-term storage of *Ascosphaera aggregata* and *Ascosphaera apis*, pathogens of the leafcutting bee (*Megachile rotundata*) and the honey bee (*Apis mellifera*). J. Invertebr. Pathol. 101: 157-160.

Jensen, A.B., Pedersen, B.V., and J. Eilenberg. 2009b. Differential susceptibility across honey bee colonies in larval chalkbrood resistance. Apidologie 40: 524-534.

Johnson, E.A., and A.E. Larson. 2005. Lysozyme, pp. 361–387. In P.M. Davidson, J.N. Sofos and A.L. Branen (Eds.), Antimicrobials in food, 3rd ed. Taylor & Francis, Boca Raton, FL.

Klein, A.M., Vaissière, B., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., and T. Tscharntke. 2007. Importance of crop pollinators in changing landscapes for world crops. Proc. R. Soc. Lond. B, Biol. Sci. 274: 303-313.

Koenig, J.P., Boush, G.M., and E.H. Erickson. 1987. Effects of spore introduction and ratio of adult bees to brood on chalkbrood disease in honeybee colonies. J. Apic. Res. 26: 191-195.

Lapidge, K., Oldroyd, B., and M. Spivak. 2002. Seven suggestive quantitative trait loci influence hygienic behavior of honey bees. Naturwissenschaften 89: 565-568.
Lesnierowski, G., and J. Kijowski. 2007. Lysozyme, pp. 33-40. In R. Huopalahti, R. López-Fandiño, M. Anton and R. Schade (Eds.), Bioactive egg compounds. Springer-Verlag, Heidleberg, Berlin.

Litwack, G., and A.L.N. Prasad. 1962. Development of lysozyme resistance in *Micrococcus lysodeikticus* and *Sarcina lutea*. Nature 196: 143-145.

Lopera, D., Aristizabal, B.H., Restrepo, A., Cano Luz, E., and Á González. 2008. Lysozyme plays a dual role against the dimorphic fungus *Paracoccidioides brasiliensis*. Rev. Inst. Med. Trop. São Paulo 50: 169-175.

Lumbsch, H.T. and S.M. Huhndorf (eds). 2007. Outline of Ascomycota. Myconet 13:1 –58.

Maassen, A. 1913. Weitere Mitteilungen uber der seuchenhaften Brutkrankheiten der Bienen [Further communication on the epidemic brood disease of bees]. Mitt. Biol. Anst. Land.-Fortsn. 14:48–58.

Maassen, A. 1916. Über bienenkrankheiten [On bee diseases]. Mitt. Biol. Anst. Land.-Fortsn. 16: 51-58.

Martel, A.C., Zeggane, S., Drajnudel, P., Faucon, J.P., and M. Aubert. 2006. Tetracycline residues in honey after hive treatment. Food Addit. Contam. 23: 265-273.

Masschalck, B., and C.W. Michiels. 2003. Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. Crit. Rev. Microbiol. 29: 191-214.

Masterman, R., Ross, R., Mesce, K., and M. Spivak. 2001. Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis mellifera* L). J. Comp. Physiol. A 187: 441-452.

Mattila, H.R., and T.D. Seeley. 2007. Genetic diversity in honey bee colonies enhances productivity and fitness. Science 317: 362-364.

McKee, B. 2003. Prevention of residues in honey: A future perspective. Apiacta, 38: 173-177.

Mehr, Z., Menapace, D.M., Wilson, W.T., and R.R. Sackett. 1976. Studies on the initiation and spread of chalkbrood within an apiary. Am. Bee J. 116: 266-268.

Melathopoulos, A., Nelson, D., and K. Clark. 2004. High velocity electron-beam radiation of pollen and comb for the control of *Paenibacillus larvae* subspecies *larvae* and *Ascosphaera apis*. Am. Bee J. 144: 714-720.

Menapace, D.M., and P. Hale. 1981. Citral and a combination of sodium propionate and potassium sorbate did not control chalkbrood. Am. Bee J. 121: 889-891.

Moritz, R.F.A., and S. Fuchs. 1998. Organization of honeybee colonies: characteristics and consequences of a superorganism concept. Apidologie 29: 7-21.

Morse, R.A., and K. Flottum (eds). 1997. Honey bee pests, predators and diseases, 3rd ed. A.I. Root Company, Medina, OH.

Nelson, D.L., and T.A. Gochnauer. 1982. Field and laboratory studies on chalkbrood disease of honeybees. Am. Bee J. 122: 29–34.

Neumann, P., and C. Carreck. 2010. Honey bee colony losses: a global perspective. J. Apic. Res. 49: 1-6.

Oldroyd, B.P., and J.H. Fewell. 2007. Genetic diversity promotes homeostasis in insect colonies. Trends Ecol. Evol. 22: 408-413.

Oxley, P.R., Spivak, M., and B. Oldroyd. 2010. Six quantitative trait loci influence task thresholds for hygienic behaviour in honeybees (*Apis mellifera*). Mol. Ecol. 19: 1452-1461.

Özcengiz G., and N.G. Alaeddinoglu. 1991. Bacilysin production and sporulation in *Bacillus subtilis*. Curr. Microbiol. 23: 61-64.

Pimentel, D., Wilson, C., McCullum, C., Huang, R., Dwen, P., Flack, J., Tran, Q., Saltman, T., and B. Cliff. 1997. Economic and environmental benefits of biodiversity. BioScience 47: 747-757.

Peng ,Y.S.C., Mussen, E., Fong, A., Montague, M.A., and T. Tyler. 1992. Effects of chlortetracycline of honey bee worker larvae reared in vitro. J. Invertebr. Pathol. 60: 127-133.

Proctor, V.A., and F.E. Cunningham. 1988. The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. Crit. Rev. Food Sci. Nutr. 26: 359-395.

Proctor, V.A., and F.E. Cunningham. 1993. The antimicrobial properties of lysozyme alone and in combination with other additives in vitro and in selected meat products. J. Rapid Meth. Automat. Microbiol. 1: 315-328.

Puerta, F., Flores, J.M., Ruiz, J.A., Ruiz, J.M., and F. Campano. 1999. Fungal diseases of the honey bee (*Apis mellifera* L.). Options Medierraneennes Series B 25: 61-68.

Rothenbuhler, W.C. 1964. Behaviour genetics of nest cleaning honeybees. IV. Responses of F1 and backcross generations to disease killed brood. Am. Zool. 4: 111-123.

Rundassa, D.B. 2006. Chalkbrood in Ethiopa. Bees for Development Journal 78: 8.

Russel, A.D. 1991. Mechanisms of bacterial resistance to non-antibiotics: food additives and food and pharmaceutical preservatives. J. Appl. Bacteriol. 71: 191-201.

Seeley, T.D. 1989. The honey bee colony as a superorganism. Am. Sci. 77: 546-553.

Shimanuki, H., and D. A. Knox. 1997. Summary of control methods, pp 493-512. In R.A. Morse and K. Flottum (eds.), Honey bee pests, predators and diseases, 3rd ed. A.I. Root Company, Medina, OH.

Skou, J.P. 1988. More details in support of the class *Ascosphaeromycetes*. Mycotaxon 31: 191-198.

Spiltoir, C.F. 1955. Life cycle of *Ascosphaera apis* (*Pericystis apis*). Am. J. Bot. 42:501-508.

Spiltoir, C.F., and L.S. Olive. 1955. A reclassification of the genus *Pericystis* Betts. Mycologia 47: 238-244.

Spivak, M., and M. Gilliam. 1993. Facultative expression of hygienic behavior of honeybees in relation to disease resistance. J. Apic. Res. 32: 147-157.

Spivak, M., and G.S. Reuter. 1998. Performance of hygienic honey bee colonies in a commercial apiary. Apidologie 29: 291-302.

Strominger, J.L., and D.J. Tipper. 1974. Structure of bacterial cell walls: the lysozyme substrate, pp. 169–184. In: E.F. Osserman., R.E. Canfield and S. Beychok (eds), Lysozyme. Academic Press, New York, NY.

Swanson, J.A.I., Torto, B., Kells, S.A., Mesce, K.A., Tumlinson, J.H., and M. Spivak. 2009. Odorants that induce hygienic behavior in honeybees: identification of volatile compounds in chalkbrood-infected honeybee larvae. J. Chem. Ecol. 35: 1108-1116.

Takala, T.M., and P.E.J. Saris. 2007. Nisin: past, present, and future, pp181-213. In: M.A. Riley and O. Gillor (eds.), Research and applications in bacteriocins. Cromwell Press, Trowbridge, Wiltshire, UK.

Tarpy, D.R. 2003. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. Proc. R. Soc. Lond. B 270: 99-103.

Tarpy, D.R., and D.I. Nielsen. 2002. Sampling error, effective paternity, and estimating the genetic structure of honey bee colonies (Hymenoptera: Apidae). Ann. Entomol. Soc. Am. 95: 513-528.

Tarpy D.R., and T.D. Seeley. 2006. Lower disease infections in honeybee (*Apis mellifera*) colonies headed by polyandrous vs monandrous queens. Naturwissenschaften 93: 195-199.

Theantana, T., and P. Chantawannakul. 2008. Protease and ß-N acetylglucosaminidase of honey bee chalkbrood pathogen *Ascosphaera apis*. J. Apic. Res. 47: 68-76.

Torchio, P.F. 1992. Effects of spore dosage and temperature on pathogenic expressions of chalkbrood syndrome caused by *Ascosphaera torchioi* within larvae of *Osmia lignaria propinqua* (Hymenoptera: Megachilidae). Environ. Entomol. 21: 1086-1091.

Toumanoff, C. 1951. Les maladies des abeilles [Diseases of bees]. Rev. Fr. Apic. Num. Spec. 68: 325.

Vandenberg, J.D., and W.P. Stephen. 1982. Etiology and symptomology of chalkbrood in the alfalfa leafcutting bee, *Megachile rotundata*. J. Invertebr. Pathol. 39:133-137.

vanEngelsdorp, D., and M.D. Meixner. 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J. Invertebr. Pathol. 103: S80-S95.

Virgil (70-19B.C.). 2002. Georgics IV (as translated by A.S. Kline). http://www.poetryintranslation.com/PITBR/Latin/VirgilGeorgicsIV.htm.

Winston, M.L. 1987. The biology of the honey bee. Harvard University Press, Cambridge, Mass.

Winston, M.L. 1992. The honey bee colony: life history, pp73-101. In J.M. Grant (ed.), The hive and the honey bee. Dadant and Sons Inc., Hamilton, IL.

Whitfield, C.W., Behura, S.K., Berlocher, S.H., Clark, A.G., Johnston, J.S., Sheppard, W.S., Smith, D.R., Suarez, A.V., Weaver, D., and N.D. Tsutsui. 2006. Thrice out of Africa: ancient and recent expansions of the honey bee, *Apis mellifera*. Science 314: 642-645.

Chapter 2: The Acute and Chronic Oral Toxicity of Lysozyme-HCl and Nisin to Adult Worker Bees and the *in Vitro* Efficacy of Lysozyme-HCl for the Treatment of Chalkbrood Disease

Introduction

Chalkbrood is an economically important disease of honey bee larvae (*Apis mellifera* L.) caused by the heterothallic fungus *Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir (Spiltoir and Olive 1955). Losses attributed to chalkbrood have been estimated to vary from 1-37% of honey yields and up to 49% reduced foraging capacity (Heath 1982a; Yakobsen et al. 1991; Zaghoul et al. 2005). Although first described in 1913 (Maassen 1913 as cited in Spiltoir and Olive 1955), chalkbrood was already considered a common European honey bee disease at that time (Maassen 1916 as cited in Spiltoir and Olive 1955) at that time. Chalkbrood is now distributed worldwide (Ellis and Munn 2005) and has been present in North America since at least 1968 (Baker and Torchio 1968). The worldwide spread of chalkbrood may be explained by the global export of honey bees, bee products, and equipment contaminated with *A. apis* spores, where they can remain viable for at least 15 years (Toumanoff 1951 as cited in Jensen et al. 2009a; Heath 1985).

The spores of *A. apis* are ingested by honey bee larvae during feeding. Once consumed, the spores germinate within the lumen of the midgut, particularly at the hind end (Bamford and Heath 1989; Gilliam and Vandenberg 1997; Aronstein

and Murray 2010). Germination is most likely activated by CO_2 which is produced by the larvae during respiration (Heath and Gaze 1987) however, mycelia cannot grow anaerobically (Heath 1982b). Larvae three- to four-days old are more susceptible to chalkbrood infection than younger larvae (Aronstein and Murray 2010). This may be because the mycelia are not subjected as long to the anaerobic conditions of the digestive tract in older aged larvae (Bailey 1968). Larval chilling, suggested to increase the amount of oxygen in the tissues, also facilitates mycelial growth and has been shown to increase incidence of chalkbrood infection and mummification (Bailey 1967; Flores et al. 1996). The vegetative growth of *A. apis* breaches the peritrophic membrane lining the larval midgut through enzymatic degradation and mechanical pressure exerted by the growing hyphae (Bamford and Heath 1989; Alonso et al. 1993). The hyphae proliferate in the hemocoel and grow through the cuticle spreading from the posterior end until the entire larva is covered in fungal growth (Chorbiński 2004; Aronstein and Murray 2010). The diseased larva, overgrown with hyphae, hardens into a chalk-like mummy in its cell, which is then uncapped and removed by adult worker bees (Gilliam and Vandenberg 1997).

Chalkbrood mummies can be either white or gray-black. The black colour is the result of the presence of spore producing fruiting bodies that are brown to black when mature (Anderson and Gibson 1998) and occurs when a diseased larva is simultaneously infected with both + and – strains of *A. apis* that have mated. A single chalkbrood mummy can contain as many as 10^8-10^9 spores (Nelson and

Gochnauer 1982). As *A. apis* is heterothallic, diseased larvae are white if a single strain is more successful in colonizing a larva (Christensen and Gilliam 1983). These remain unmated, or if mated, do not produce fruiting bodies before the cadaver hardens (Aronstein and Murray 2010).

Despite numerous studies of possible chemical treatments (Hornitzky 2001), there is no registered chemotherapeutic treatment available for chalkbrood disease in North America. In absence of chemical controls, several techniques are available to apiculturists to mitigate the effects of chalkbrood disease. Comb replacement or equipment disinfection can be used to reduce the level of *A. apis* spores in the colony (Gochnauer and Margetts 1980; Nelson and Gochnauer 1982). To minimize brood chilling, beekeepers can avoid placing apiaries in low-lying, cool locations or by strengthening weak colonies with the addition of adult worker bees to increase adult to brood ratio (Heath 1982a). Genetic variation for chalkbrood susceptibility in honey bee populations has been documented (Milne 1983; Jensen et al. 2009b). Queen bees in heavily infected colonies are often removed and replaced with new queens to establish a worker population that is more resistant to chalkbrood disease (Bailey and Ball 1991). Honey bee workers which engage in “hygienic” behaviours, uncapping and removing diseased larvae prior to sporulation, have been shown to be more resistant to chalkbrood (Spivak and Gilliam 1993). However, the genes for hygienic behaviour are postulated to be recessive, multi-locus, and may be difficult to maintain without continuous selective breeding (Rothenbuler 1964; Moritz 1988; Spivak and Reuter 1998).

The difficulty in maintaining disease-resistant stock and the high cost of queen and comb replacement has driven much of the research on possible chemical treatments. These treatments must be non-toxic to both honey bees and humans as well as cheaper than the yield loss attributed to chalkbrood (Menapace and Hale 1981; Heath 1982a). It is also critical to establish that a treatment for chalkbrood will have no negative effects on the adult worker population as treatments for larval diseases in honey bees are usually transmitted through adult workers upon whom they rely for food. For example, the antibiotics oxytetracycline (OTC) and tylosin tartrate, which are used to treat the bacterial disease American foulbrood (*Paenibacillus larvae* (sensu Genersch et al. 2006)), are administered to the colony in the form of pollen patties, sugar dustings, or sucrose syrup (Mutinelli 2003). Adult worker bees consume the treatment and the antibiotic is transferred to larvae from nurse bees during feeding. Treatments not immediately consumed are stored as honey in the colony and may persist for extended periods of time (Argauer and Moats 1991; Thompson et al. 2007).

As concerns over antibiotic residues in food products such as honey increase, investigations into the control of chalkbrood using natural or food grade products have been of great interest (Davis and Ward 2003). Two compounds, lysozyme-HCl and nisin, already in use in food products (Johnson and Larson 2005; Thomas and Delves-Broughton 2005) have been shown to have putative activity against the fungal pathogen *A. apis* in laboratory studies (Neova Technologies Inc.,

unpublished data). Lysozyme-HCl is a broad spectrum antimicrobial extracted from egg albumen and commonly used in wine and cheese making for the control of Gram- positive bacteria (Johnson and Larson 2005). Similarly nisin, a fermentation product of *Lactococcus lactis* (Lister 1983) Schleifer et al. 1986, is also active against Gram-positive bacteria and frequently used as a food preservative (Abee and Delves-Broughton 2003). Although most active against Gram-positive bacteria, the ability of both nisin and lysozyme-HCl to suppress *A. apis* in culture suggests they may be candidates for control of chalkbrood disease in the field. Nevertheless, successful inhibition of *A. apis* in artificial media does not always translate into an efficacious treatment *in vivo*. Moreover, the acute and chronic effects of consumption of lysozyme-HCl and nisin on honey bees is not known.

The objectives of this study were to assess short and long-term effects of lysozyme-HCl and nisin consumption on adult worker bees using acute and chronic oral toxicity assays, and determine if these compounds could suppress chalkbrood disease in larvae reared *in vitro*.

Methods

Acute Oral Toxicity

The acute oral toxicity of lysozyme-HCl (inovapure^{TM} 300, Neova Technologies, Abbotsford, BC) or nisin (Novasin^{TM}, Neova Technologies, Abbotsford, B.C.) to adult worker bees was determined using cages of field-collected bees, treated and

incubated (Incubator Model # L-30B-L, Percival Scientific, Boone, IA) in the laboratory at 34±1°C, 60±5% RH. Frames of sealed brood, collected from colonies at the AAFC Beaverlodge Research Farm, were held overnight at 34°C. All workers emerging within 36 h were paint-marked on the dorsal side of the thorax and returned to a single colony to be collected seven days later. Wooden cages (7.6 x 11.4 x 8.9 cm; outside dimensions) of 30 adult workers, aged 7-9 d, were fed target doses (0, 100, 200, 400, 800, 1600, 6400 µg per bee) of lysozyme-HCl in a 50% (w/v) sucrose solution and monitored over a period of 72 h (EPPO 1992). Each dose was replicated twice on three different dates (14, 19 July and 3 August 2005) for a total of six replicates. Similarly, adult worker bees were fed target doses of nisin (0, 12.5, 25, 50, 100, 200, 400, 800, 1600, 3200, 6400 µg per bee) on 3 different dates (14 July, 30 August and 12 October 2006); however, not all doses could be administered on each date. Between four and 10 replicates were run for each dose of nisin.

Prior to administration of each treatment, bees were starved for 2 h to ensure maximum uptake of the test substances, and then allowed to feed for 4 h before removing the sucrose solution containing lysozyme-HCl or nisin. Bees were then fed 50% (w/v) sucrose solution *ad libitum* for the remainder of the test. Fresh water was provided throughout the entire experiment. Treatments and water were delivered in 2 mL borosilicate glass vials (KG-33, Kimble,Vineland, NJ) suspended at the top of the cage. Mortality among all the treatment groups was compared to the dose-related response of a highly toxic reference compound,

dimethoate (Sigma, St. Louis, MO), (0.0875, 0.175, 0.35 and 0.7 µg per bee), and in the lysozyme-HCl trials, to the antibiotic oxytetracycline (Sigma, St. Louis, MO) (100, 200, 400, 800, 1600, 6400 µg per bee). In cases where the treatment was not fully consumed the dose each bee received was calculated to the amount actually consumed by weighing the feeding vials pre and post treatment. As there were no significant differences in control mortality among dates, trials were combined and the relationships between dose and mortality at 24, 48 and 72 h were modeled using logistic regression allowing the LD_{50} to be estimated for each compound (SAS Institute Inc. 2009).

Chronic Oral Toxicity

The chronic oral toxicity of lysozyme-HCl and nisin was determined by continuously feeding cages (7.6 x 11.4 x 5.1 cm; outside dimensions) containing 100 newly- emerged worker honey bees a range of concentrations of lysozyme-HCl in 50% (w/v) sucrose solution (Figure 2.1). Newly-emerged worker bees were collected from sealed brood frames that were removed from colonies at AAFC Beaverlodge Research Farm and held overnight at 34°C. Cages of newly emerged workers less than 24 h-old were fed a mixture of candy [75% icing sugar and 25% honey (w/w)] and water *ad libitum* over the first 48 h, and also had a pollen substitute patty (Bee Pro®, Mann Lake Ltd, Hackensack, MN) provided for 8 d according to the methods described by Dechaume Moncharmont et al. (2003). At 48 h, the candy was removed and the treatments of lysozyme-HCl or nisin in a 50% (w/v) sucrose solution were applied (0, 1.6, 16, 160 µg/mL). Treatments

were fed *ad libitum* and fresh treatments were prepared and administered daily, except for weekends. Cages were incubated (Incubator Model # L-30B-L, Percival Scientific, Boone, IA) at 34±1°C for the duration of the trial. Mortality was monitored each day except for weekends for 21 d. The mean proportion of workers surviving per cage at 7, 14, and 21 d for each dose was compared using a one-way analysis of variance (ANOVA); differences in survival among treatments were compared to the untreated control using Dunnett's test (SAS Institute Inc. 2009).

In vitro Larval Rearing Assay

The *in vitro* larval rearing assay was modified from the protocol described by Peng et al. (1992) to evaluate the effects of feeding lysozyme-HCl to larval honey bees inoculated with *A. apis* spores. Honey bee queens were excluded on an empty brood frame in a single frame excluder for 24 hours to provide larvae of a known age (Figure 2.2). For each treatment three replicates of honey bee larvae (n=30-33) less than 24 h-old were grafted into sterile Petri dishes (60 x 15mm, Falcon, Becton Dickinson and Company, Franklin Lakes, NJ) containing an excess of basic larval diet (BLD) and reared to adulthood in an incubator (Percival, Model # L-30B-L) set to 34±1° C, 90±5% RH. The diet contained 4.2 g lyophilized royal jelly (Planet Bee, Vernon, BC, Canada), 0.6 g fructose (Sigma, St. Louis, MO), 0.6 g dextrose (Difco Laboratories, Detroit, MI), 0.2 g granulated yeast extract (EMD Chemicals, Gibbstown, NJ) and 14.4 g sterile reverse-osmosis water. The larvae were transferred onto fresh food 72 h after the initial graft and

monitored daily until defecation (indicated by presence of uric acid crystals and yellow nitrogenous waste) occurred, at which time they were moved to pupation trays (24-well tissue culture plates lined with Kimwipe® tissues). These were examined daily until adult emergence (Figure 2.3). At the time of transfer to the pupation trays (3.4 mL well, BD Falcon, New York, NY) larvae were removed from the incubator and chilled to 28-30°C for 1 h to increase infection and mummification (Bailey 1967; Puerta et al. 1994). Larvae in the inoculated treatment groups were fed 1.0 x 10^8 *A. apis* spores (approximating the number of spores found in a single mummy) mixed into their BLD 72 h after grafting and fed doses of lysozyme-HCl [0, 0.75, 1.5, 3% (w/w)] in the BLD throughout the entire assay. Larvae were inoculated after 72 hours as previous research has shown that larvae are most susceptible to chalkbrood disease when they are 3-4 d old (Bailey 1967). Uninoculated, untreated larvae in the control group were fed clean BLD, without *A. apis* spores or lysozyme-HCL, until defecation. The spores used in the inoculation were collected from diseased honey bee colonies in the Beaverlodge area. Black chalkbrood mummies were homogenized in 1 mL sterile water using a glass/glass tissue homogenizer (Kontes, Vineland, NJ) and the spores enumerated using a Petroff Hauser counting chamber and phase contrast microscope. Post inoculation, all dead larvae or pupae were observed for symptoms of chalkbrood infection and visible *A. apis* growth. Symptoms included hardened cream coloured larval bodies, white hyphae penetrating the larval body usually at the posterior end, larvae engulfed with white floccose mycelial growth (Figure 2.4, 2.5) and eventual mummification (Bamford and

Heath 1989; Anderson and Gibson 1998). Mycelial growth was examined microscopically for evidence of dichotomous branching.

The proportion of total larvae per tray for each category (pre-defecation mortality, post-defecation mortality, adult emergence, and larvae exhibiting symptoms of chalkbrood disease) was compared among concentrations using one-way ANOVA and *a posteriori* comparisons, (Tukey-Kramer HSD; JMP 7.01, SAS Institute, Gary, North Carolina).

Results

Acute Oral Toxicity

Lysozyme-HCl and nisin were not acutely toxic to adult worker honey bees (Table 2.1). In fact, the highest target doses tested (6400μg per bee) failed to result in significant adult mortality within 72 h. Oxytetracycline had a 24-h LD_{50} of 3448 μg per bee and after 72 h, the LD_{50} decreased to 1130 μg per bee. In contrast, dimethoate was highly toxic with a LD_{50} of 0.29 μg per bee and did not change appreciably throughout the 72 h test period.

Chronic Oral Toxicity

Chronic consumption of the highest treatment concentration of lysozyme-HCl (160 μg/mL) by adult workers resulted in complete mortality within 21 d (Table 2.2). Cumulative mortality at the highest dose consumed was significantly different from the untreated control at days 14 ($F = 870.40$; df = 3,7; $P < 0.0001$)

and 21 (F = 581.73; df = 3,7; P < 0.0001) but not at day 7 (F = 3.81; df = 3,7; P = 0.0658). Cumulative mortality of the median dose of lysozyme-HCl was significantly greater than the untreated controls only at day 21. Cages of adult bees fed 16 μg/mL of lysozyme-HCl daily, experienced 11.6% mortality compared to 3.1% mortality in the control cages. Mortality was slighter higher in cages consuming the lowest concentration (6.2%) than the control cages (3.1%) on day 21, but was not significantly different.

Adult workers consuming nisin at all concentrations exhibited significantly greater mortality than the untreated control at days 7 (Table 2.3; F = 214.04; df = 3,7; P < 0.0001), 14 (F = 22.95; df = 3,7; P < 0.0005), and 21 (F = 25.12; df = 3,7; P = 0.0004). Complete mortality occurred by day 7 for the highest concentration of nisin tested (160 μg/mL) and by day 21 for the median dose (16 μg/mL).

In vitro Larval Rearing Assay

Pre-defecation mortality in larvae inoculated with 1 x 10^8 *A. apis* spores/mL BLD was significantly different among treatment groups (Figure 2.6; F = 111.45; df = 4,10; P < 0.0001). Inoculated larvae fed concentrations of 0.75, 1.5, and 3% lysozyme-HCl demonstrated a significant decrease in larval (pre-defecation) mortality compared with the inoculated control and did not differ from the untreated, inoculated control. There was a slight increase in pupal (post defecation) mortality (Figure 2.6; F = 4.90; df = 4,10; P = 0.019) at 3% lysozyme-

HCl compared to the other treatments. Adult emergence success for inoculated treatment groups fed either 0.75, 1.5 or 3% of lysozyme-HCl and the uninoculated controls was similar and ranged between 60-70% and was significantly greater than the inoculated controls (Figure 2.6; $F=35.66$, d.f.= 4,10, $P<0.0001$). Mortality (pre and post defecation) attributed to chalkbrood infection was significantly higher in the inoculated control (89%) compared to the other treatment groups (Figure 2.7; $F=145.87$, d.f.= 4,10, $P<0.0001$). Lysozyme-HCl in the diet of inoculated larvae did not completely suppress chalkbrood symptoms and increasing the concentration of lysozyme-HCl did not reduce the incidence of chalkbrood as all three treatments (0.75, 1.5, 3.0%) had similar levels of mortality attributed to chalkbrood (20-23%) (Figure 2.7).

Discussion

Lysozyme-HCl and nisin were not acutely toxic at the highest dose tested (6400 μg per bee) even after 72 hours to adult honey bee workers, as classified by international standards (Felton et al. 1986). Oxytetracycline, an antibiotic already in use in North American honey bee colonies as a treatment for American foulbrood, was similarly found to be non-toxic (Table 2.1). Differences in LD_{50} values at 72 h for OTC from previous acute oral toxicity studies (Alippi et al. 1999) may be a result of different incubation temperatures. The majority of acute toxicity studies are conducted at temperatures of 25°C and not at internal hive temperatures of 33-35°C (EPPO 1992). As a potential treatment for chalkbrood, lysozyme-HCl and nisin would be administered inside the hive where they would

be consumed by larvae. Therefore the oral toxicity studies were conducted at 34°C. At 25°C, oxytetracycline has a 72 h LD_{50} of 230 μg per bee (Alippi et al. 1999) whereas at 34°C, the LD_{50} was 1130 μg per bee (Table 2.1). In honey, tetracycline (structurally similar to oxytetracycline) degraded twice as fast at 35°C than at 20°C (Martel et al. 2006) and may explain the increase in amount required after 72 h to kill 50% of the sample population. The 24 h LD_{50} of 0.29 μg per bee for dimethoate, a toxic standard for honey bee acute oral toxicity, was within the range of acceptable values of 0.10-0.33 μg per bee (EPPO 1992) and did not show any latent effects after 72 h as was also demonstrated by Gough et al. (1994).

It has previously been reported that a honey bee worker fed *ad libitum* consumes on average 12-46 μL per day (Suchail et al. 2001; Dechaume Moncharmont et al. 2003; Decourtye et al. 2003; Schmuck 2004). Based on these estimates, it was calculated that individual bees in the chronic oral toxicity trials consumed between 0.0192 to 73.6 μg lysozyme-HCl or nisin per day. At the lowest concentration tested (1.6 μg/mL), over the course of 21 days, individual honey bees would have consumed between 0.4-1.5 μg of lysozyme-HCl or nisin and at the highest concentration (160 μg/mL) between 40-150 μg. The detrimental effects of chronic consumption increased with concentration of both lysozyme-HCl and nisin (Tables 2.2, 2.3). The only treatment that did not differ significantly from the untreated control after 21 days was the lowest concentration of lysozyme-HCl (1.6 μg/mL) evaluated. The estimated daily dose, 0.4-1.5 μg of lysozyme-HCl, actually consumed by honey bees fed 1.6 μg/mL lysozyme-HCl in

sugar syrup is much lower than the value obtained by Maistrello et al. (2008). In cage trials, newly-emerged bees fed 2.5 mg/g lysozyme in sugar candy, consumed 122.5 μg of lysozyme daily for 11 days without significant mortality when compared to control bees. However, mortality in the control cages in that study was greater than 50% (Maistrello et al. 2008). The increase in toxic effects of nisin compared with lysozyme-HCl may be attributed to the formulation of Novasin^{TM}, which is composed of 77.5% sodium chloride (O'Connor et al. 2007). Sodium chloride is toxic to caged bees at levels as low as 0.125% (w/v) in sucrose syrup (Barker 1977). Based on the potential for toxicity in commercial preparations of nisin, it was eliminated as a potential treatment for brood diseases at the colony level.

To evaluate the effects of lysozyme-HCl on chalkbrood infection in larvae, modifications to the *in vitro* larval rearing protocol established by Peng et al. (1992) for the assessment of American foulbrood were made. Inoculation of larvae with *A. apis* spores 72 hours after the initial transfer, chilling of the larvae for 1 hour post defecation, and elimination of daily larval transfer reduced the labour involved in the assay and enabled consistent infection of worker larvae with chalkbrood disease. The mortality rate attributed to chalkbrood disease in untreated inoculated larvae was extremely high (89±2.8%) and only 1 larva in the uninoculated control treatments died as a result of chalkbrood infection (Figure 2.7). The presence of chalkbrood in the uninoculated controls may be due to pre-existing *A. apis* spores in the original source colony the larvae were collected

from as it has been documented that spores can be present in asymptomatic colonies (Gilliam 1986).

Inoculated larvae treated with lysozyme-HCl at the lowest concentration tested (0.75% BLD) demonstrated levels of adult emergence equal to that of the uninoculated, untreated control. Repression of chalkbrood infection was not complete at that concentration, but a four-fold increase of lysozyme-HCl (3.0% BLD) did not decrease the proportion of larvae exhibiting clinical symptoms of chalkbrood disease. This demonstrates that although lysozyme-HCl is highly fungistatic it does not completely protect larvae from the effects of chalkbrood. As pupal mortality increased and emergence success decreased when inoculated larvae were treated with 3% lysozyme-HCl, it is possible that at concentrations higher than 1.5% BLD, lysozyme-HCl may have latent toxic effects.

The mechanism of action by which lysozyme-HCl inhibits *A. apis* growth has not been characterized but the ability of lysozyme to inhibit fungi has been established and attributed to both the structure, which contains amphipathic helical regions that can disrupt cell membranes (Düring et al. 1999) and weak enzymatic chitinase activity that degrades fungal cell walls (Johnson and Larson 2005). In *Paracoccidioides brasiliensis* (Splend.) F.P. Almeida hen egg white lysozyme plays a dual role, causing ultra-structural changes in the conidia (asexual spores) and inhibiting conidium to yeast transitions and yeast budding (Lopera et al. 2008). Although a conidial state is absent in *A. apis*, these findings

suggest that lysozyme-HCl could have multiple mechanisms of action which affect both the spore and vegetative stages. As well, multiple mechanisms of action against *A. apis* may mean that evolution of resistance by the fungal pathogen to lysozyme-HCl will be unlikely (Davidson and Harrison 2002).

The extremely low oral toxicity of lysozyme-HCl to adult honey bees and the effective suppression of chalkbrood disease in larvae reared in the laboratory support the use of lysozyme-HCl as a safe, efficacious control of chalkbrood. Further investigation at the colony level is needed to establish an inexpensive therapeutic dose of lysozyme-HCl for the novel treatment of chalkbrood disease in the field.

Figure 2.1. Cages (7.6 x 11.4 x 5.1 cm outside dimensions) containing 100 newly-emerged worker honey bees used in the chronic oral toxicity trials.

Figure 2.2. Single frame queen excluders. Queens were excluded on an empty brood frame for 24 h to provide an evenly-aged cohort of larvae.

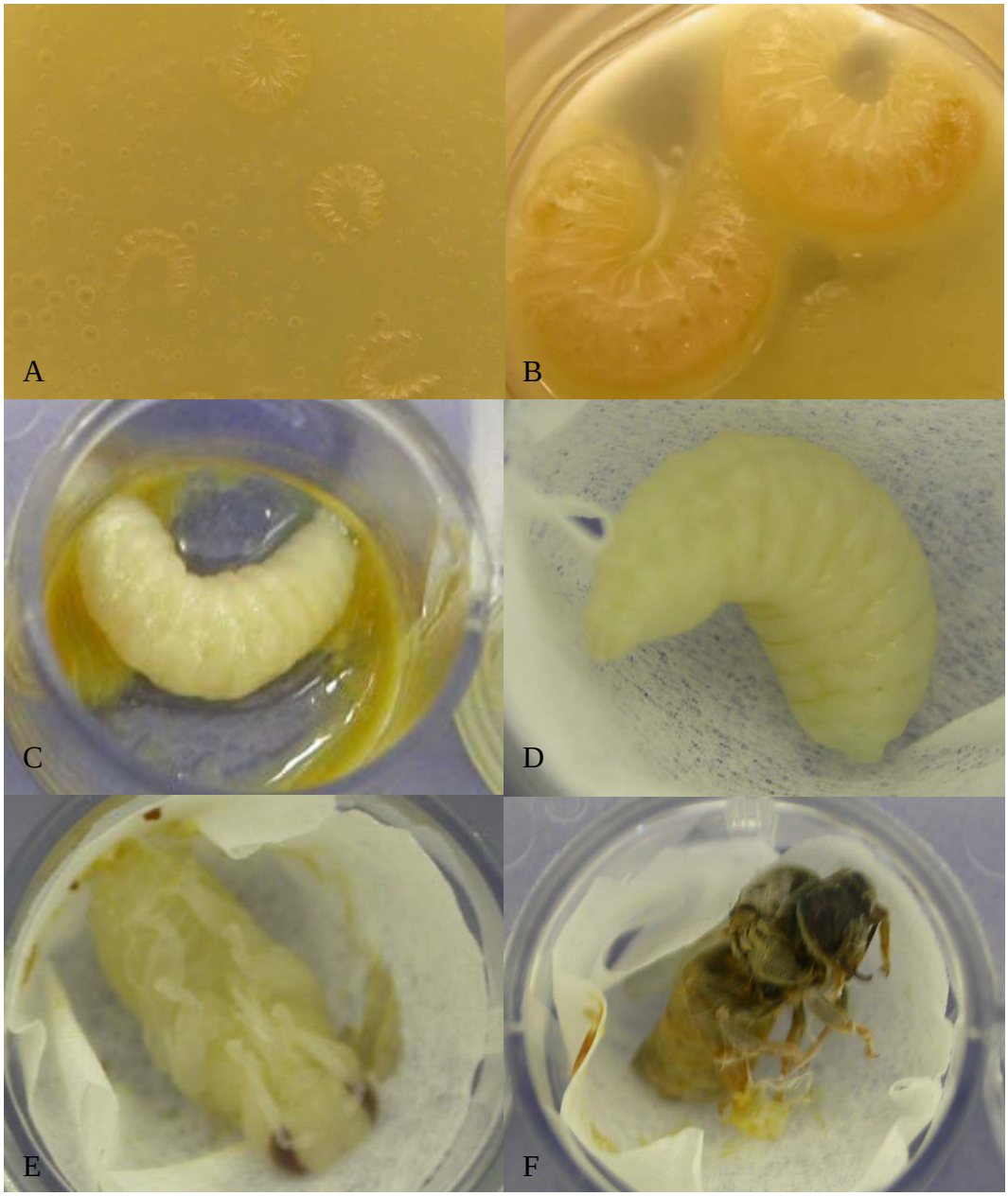

Figure 2.3. Various stages of the life cycle of honey bees reared *in vitro* (A to F): first instar larvae (A); fourth instar larvae (B); fifth instar larvae post defecation (C); prepupa (D); pupa (E); and emerging adult (F).

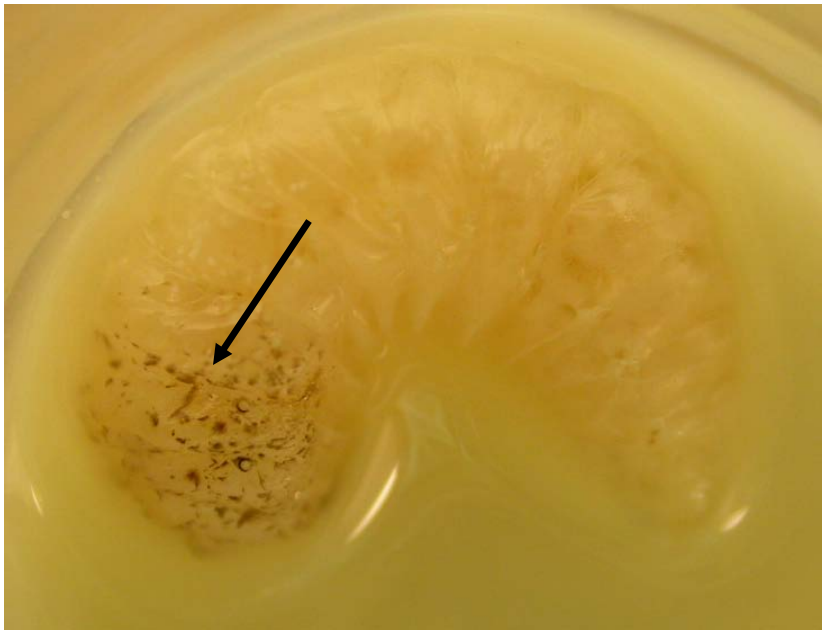

Figure 2.4. Fourth-instar larvae showing signs of fungal growth at the posterior end.

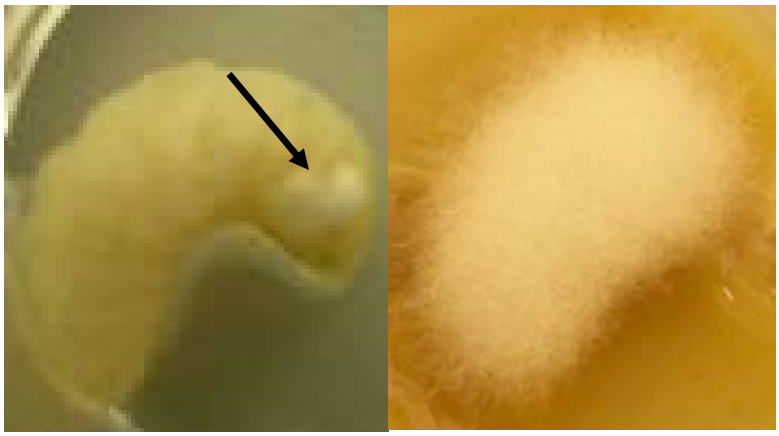

Figure 2.5. Clinical symptoms of chalkbrood (L to R): Hardened cream coloured body with white fungal mycelia starting to appear; larvae engulfed in white fluffy mycelial growth.

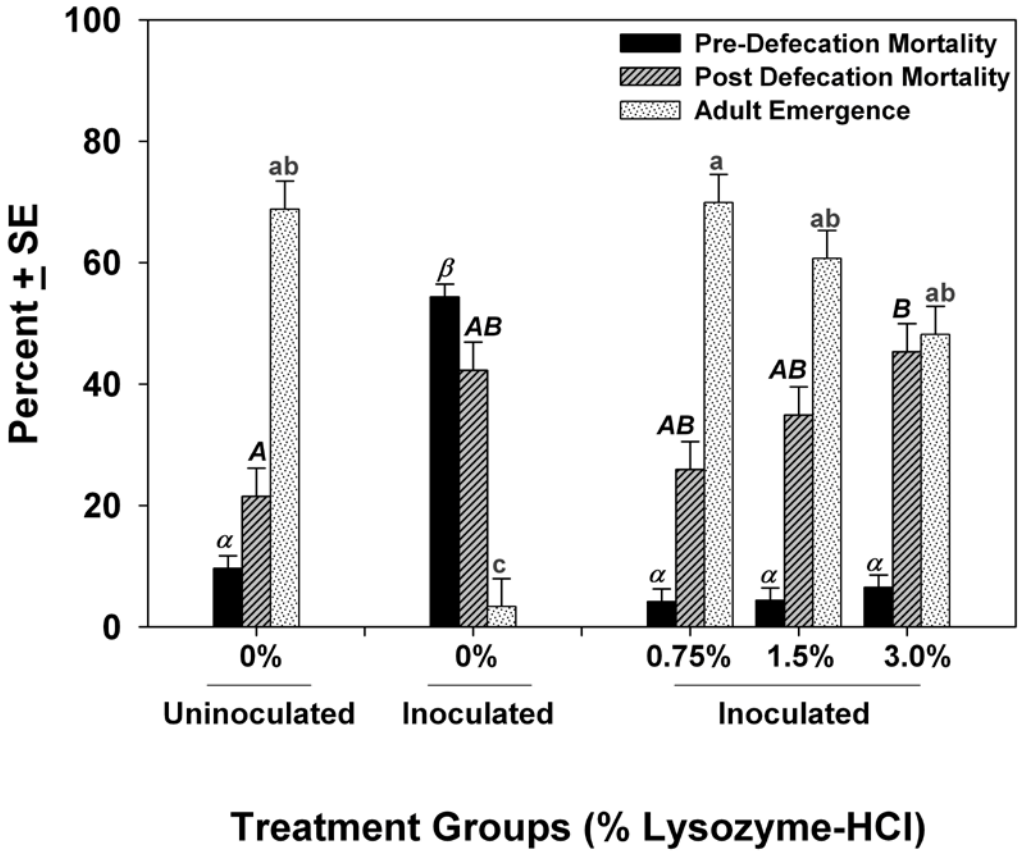

Figure 2.6. Protective effects of 0, 0.75, 1.5 and 3.0% lysozyme-HCl (inovapure™ 300) against larvae inoculated with $1x10^8$ spores *A. apis*/mL larval diet. Mean proportion of total larvae in the rearing tray dying before defecation dying after defecation and emerging as adults were all analyzed separately. Treatments with different letters are significantly different at $\alpha = 0.05$ (Tukey-Kramer HSD, n = 3 trays per treatment). For each category different letter styles were used to indicate significance: pre-defecation mortality (black, lowercase, greek letters, α); post defecation mortality (black, uppercase, italicized letters, ***A***); adult emergence (grey, lowercase letters, a).

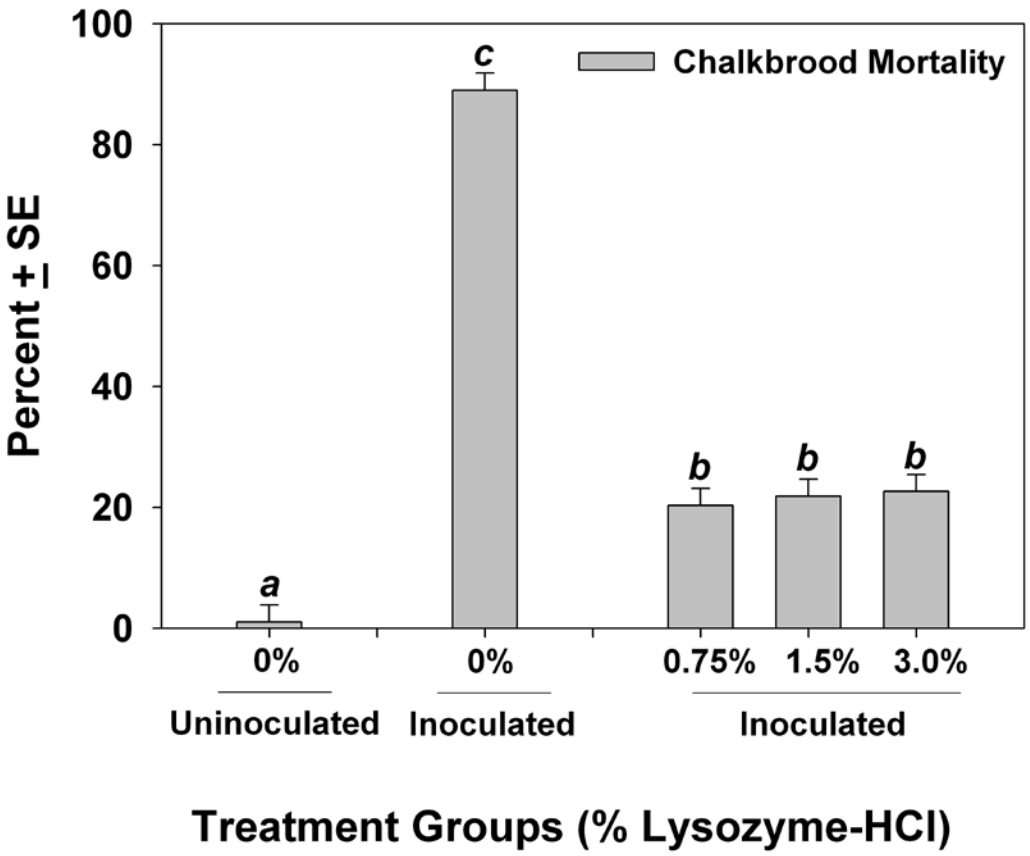

Figure 2.7. Percentage of mortality attributed to chalkbrood. Mean proportion of dead individuals before and after defecation killed by chalkbrood. Treatments with different letters are significantly different at $\alpha = 0.05$ (Tukey-Kramer HSD, n = 3 trays per treatment).

Table 2.1. Acute oral toxicity of dimethoate, oxytetracycline (OTC), lysozyme-HCl (Inovapure™ 300), and nisin (Novasin™): LD_{50} calculated using logistic regression for worker honey bees aged 7-9 d prior to exposure at 24, 48 and 72 h.

Compound	Time (h)	Intercept		Slope			LD_{50} (95% CI) (µg/bee)
		Estimate	χ^2 ($P>\chi^2$)	Estimate	χ^2 ($P>\chi^2$)		
Dimethoate	24	-1.98	203.79 (0.0001)	6.74	216.32	(0.0001)	0.29 (0.27-0.32)
Dimethoate	48	-2.06	201.95 (0.0001)	7.53	222.02	(0.0001)	0.27 (0.25-0.29)
Dimethoate	72	-2.02	191.48 (0.0001)	7.84	220.55	(0.0001)	0.26 (0.24-0.28)
OTC	24	-5.35	98.39 (0.0001)	6.97E-04	81.22	(0.0001)	3448 (3027-4046)
OTC	48	-1.00	73.63 (0.0001)	3.82E-04	32.80	(0.0001)	2626 (2105-3517)
OTC	72	-0.47	18.46 (0.0001)	4.19E-04	35.26	(0.0001)	1130 (739-1536)
Lysozyme-HCl	24	-5.35	47.66 (0.0001)	-1.69E-05	0.0032	(0.9546)	> 6400
Lysozyme-HCl	48	-4.87	56.56 (0.0001)	-1.44E-04	0.22	(0.6378)	> 6400
Lysozyme-HCl	72	-4.64	71.74 (0.0001)	-2.32E-05	0.012	(0.9126)	> 6400
Nisin	24	-2.40	207.36 (0.0001)	3.62E-04	3.59	(0.0581)	> 6400
Nisin	48	-4.98	145.78 (0.0001)	2.14E-04	1.61	(0.2048)	> 6400
Nisin	72	-4.40	234.73 (0.0001)	4.67E-04	28.75	(0.0001)	> 6400

Test of H_0; parameter = 0

Table 2.2. The chronic oral toxicity of lysozyme-HCl (Inovapure™ 300) to adult worker honey bees at 7, 14, and 21 d after exposure.

Treatments	n	Cumulative Mortality (%) ± SE		
		Day		
		7	14	21
Control	3	0.91 + 3.66	2.14 + 1.56	3.09 + 1.90
1.6 ug/mL	2	1.43 + 4.48	3.33 + 1.91	6.17 + 2.33
16 ug/mL	3	0.34 + 3.66	2.00 + 1.56	11.58 + 1.90*
160 ug/mL	3	15.32 + 3.66	95.73 + 1.56*	100 + 1.90*

*Cumulative mortality significantly different from the untreated control using Dunnett's test (P<0.05)

Table 2.3. The chronic oral toxicity of nisin (Novasin™) to adult worker honey bees at 7, 14, and 21 d. after exposure.

Treatments	n	Cumulative Mortality (%) ± SE		
		Day		
		7	14	21
Control	3	0.63 + 3.05	3.03 + 8.59	4.68 + 8.97
1.6 ug/mL	2	12.00 + 3.73*	50.85 + 10.52*	71.03 + 10.98*
16 ug/mL	3	20.48 + 3.05*	74.01 + 8.59*	100 + 8.97*
160 ug/mL	3	100 + 3.05*		

* Cumulative mortality significantly different from the untreated control using Dunnett's test (P<0.05)

Literature Cited

Abee, T., and J. Delves-Boughton. 2003. Bacteriocins – Nisin, pp. 146-178. In N. J. Russell & G. W. Gould (Eds.), Food preservatives. Kluwer Academic/Plenum Publishers, New York, NY.

Alippi, A.M., Albo, G.N., Leniz, D., Rivera, I., Zanelli, M.L., and A.E. Roca. 1999. Comparative study of tylosin, erythromycin and oxytetracycline to control American foulbrood of honey bees. J. Apic. Res. 38: 149–158.

Alonso, J.M., Rey, J., Puerta, F., Hermoso de Mendoza, J., Hermoso de Mendoza, M., and J.M. Flores. 1993. Enzymatic equipment of *Ascosphaera apis* and the development of infection by this fungus in *Apis mellifera*. Apidologie 24:383-390.

Anderson, D.L., and N.L. Gibson. 1998. New species and isolates of spore-cyst fungi (Plectomycetes: Ascosphaerales) from Australia. Aust. Syst. Bot. 11: 53-72.

Argauer, R.J., and W.A. Moats. 1991. Degradation of oxytetracycline in honey as measured by fluorescence and liquid chromatographic assays. Apidologie 22: 109–115.

Aronstein, K.A., and K.D. Murray. 2010. Chalkbrood disease in honey bees. J. Invertebr. Pathol. 103: S20-S29.

Bailey, L. 1967. The effect of temperature on the pathogenicity of the fungus *Ascosphaera apis* for larvae of the honeybee, *Apis mellifera*, pp. 162-167. *In* P.A. Van der Laan [ed], Insect pathology and microbial control. North Holland Publishing Co., Amsterdam, Netherlands.

Bailey, L. 1968. Honey bee pathology. Annu. Rev. Entomol. 13:191-212.

Bailey, L., and B.V. Ball. 1991. Honey bee pathology. Academic Press, London, UK.

Baker, G.M., and P.F. Torchio. 1968. New records of *Ascosphaera apis* from North America. Mycologia 60: 189-190.

Bamford, S., and L.A.F. Heath. 1989. The infection of *Apis mellifera* by *Ascosphaera apis*. J. Apic. Res. 28:30-35.

Barker, R.J. 1977. Considerations in selecting sugars for feeding to honey bees. Am. Bee J. 117: 76-77.

Chorbiński, P. 2004. The development of the infection of *Apis mellifera* larvae by *Ascosphaera apis*. Electron. J. Pol. Agric. Univ. 7, #03 [online]. http://www.ejpau.media.pl/volume7/issue2/veterinary/art-03.html.

Christensen, M., and M. Gilliam. 1983. Notes on the *Ascosphaera* species inciting chalkbrood in honey bees. Apidologie 14: 291–297.

Davidson, P.M. and M.A. Harrison. 2002. Resistance and adaptation to food antimicrobials, sanitizers, and other process controls. Food Technol. 56: 60-78.

Davis, C., and W. Ward. 2003. Control of chalkbrood disease with natural products. Publ. No. 03/107, Rural Industries Research and Development Corporation, Kingston ACT, Australia.

Dechaume Moncharmont, F.X., Decourtye, A., Hennequet-Hantier, C., Pons, O., and M.H. Pham-Delègue. 2003. Statistical analysis of honeybee survival after chronic exposure to insecticides. Environ. Toxicol. Chem. 22: 3088-3094.

Decourtye, A., Lacassie, E., and M.H. Pham-Delègue. 2003. Learning performances of honeybees (*Apis mellifera* L.) are differentially affected by imidacloprid according to the season. Pest. Manag. Sci. 59: 269-278.

Düring, K., Porsch, P., Mahn, A., Brinkmann, O. and Gieffers, W. 1999. The non-enzymatic microbicidal activity of lysozymes. FEBS Lett. 449: 93-100.

Ellis, J.D., and P.A. Munn. 2005. The worldwide health status of honey bees. Bee World 86: 88-101.

European and Mediterranean Plant Protection Organization/Organisation européenne et méditerranéenne pour la protection des plantes. 1992. Guidelines on test methods for evaluating the side-effects of plant protection products on honeybees. Bull. OEPP 22: 203-215.

Felton, J.C., Oomen, P.A., and J.H. Stevenson. 1986. Toxicity and hazard of pesticides to honeybees: harmonization of test methods. Bee World 67: 114-124.

Flores, J.M., Ruiz, J.A., Ruiz, J.M., Puerta, F., Busto, M., Padilla, F., and F. Campano. 1996. Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions. Apidologie 27: 185-192.

Genersch, E., Forsgren, E., Pentikäinen, J., Ashiralieva, A., Rauch, S., Kilwinski, J., and I. Fries. 2006. Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. Int. J. Syst. Evol. Microbiol. 56: 501-511.

Gilliam, M. 1986. Infectivity and survival of the chalkbrood pathogen, *Ascosphaera apis*, in colonies of honey bees, *Apis mellifera*. Apidologie 17: 93-100.

Gilliam, M., and J.D. Vandenberg. 1997. Fungi, pp. 81–110. In R.A. Morse and K. Flottum (eds.), Honey bee pests, predators and diseases, 3rd ed. A.I. Root Company, Medina, OH.

Gochnauer, T.A., and V.J. Margetts. 1980. Decontaminating effect of ethylene oxide on honeybee larvae previously killed by chalkbrood disease. J. Apic. Res. 18: 212-216.

Gough, H.J., McIndoe, E.C., and G.B. Lewis. 1994. The use of dimethoate as a reference compound on laboratory acute toxicity tests on honey bees (*Apis mellifera* L.) 1981-1992. J. Apic. Res. 33: 119-125.

Heath, L.A.F. 1982a. Development of chalk brood in a honey bee colony: a review. Bee World 63: 119-130.

Heath, L.A.F. 1982b. Chalk brood pathogens: a review. Bee World 63: 130-135.

Heath, L.A.F. 1985. Occurrence and distribution of chalkbrood disease of honey bees. Bee World 66: 9-15.

Heath, L.A.F., and B.M. Gaze. 1987. Carbon dioxide activation of spores of the chalkbrood fungus, *Ascosphaera apis*. J. Apic. Res. 26: 243-246.

Hornitzky, M. 2001. Literature review of chalkbrood–a fungal disease of honeybees. Publ. No. 01/150, Rural Industries Research and Development Corporation, Kingston ACT, Australia.

Jensen, A.B., James, R.R., and J. Eilenberg. 2009a. Long-term storage of *Ascosphaera aggregata* and *Ascosphaera apis*, pathogens of the leafcutting bee (*Megachile rotundata*) and the honey bee (*Apis mellifera*). J. Invertebr. Pathol. 101: 157-160.

Jensen, A.B., B.V. Pedersen, and J. Eilenberg. 2009b. Differential susceptibility across honey bee colonies in larval chalkbrood resistance. Apidologie 40: 524-534.

Johnson, E.A., and A.E. Larson. 2005. Lysozyme, pp. 361–387. In P.M. Davidson, J.N. Sofos, and A.L. Branen (Eds.), Antimicrobials in food, 3rd ed. Taylor and Francis, Boca Raton, FL.

Lopera, D., Aristizabal, B.H., Restrepo, A., Cano Luz, E., and Á. González. 2008. Lysozyme plays a dual role against the dimorphic fungus *Paracoccidioides brasiliensis*. Rev. Inst. Med. Trop. São Paulo 50: 169-175.

Maassen, A. 1913. Weitere Mitteilungen uber der seuchenhaften Brutkrankheiten der Bienen [Further communication on the epidemic brood disease of bees]. Mitt. Biol. Anst. Land.-Fortsn. 14:48–58.

Maassen, A. 1916. Über bienenkrankheiten [On bee diseases]. Mitt. Biol. Anst. Land.-Fortsn. 16: 51-58.

Maistrello, L., Lodesani, M., Costa, C., Leonardi, F., Marani, G., Caldon, M., Mutinelli, F., and A. Granato. 2008. Screening of natural compounds for the control of nosema disease in honeybees (*Apis mellifera*). Apidologie 39: 436-445.

Martel, A.C., Zeggane, S., Drajnudel, P., Faucon, J.P., and M. Aubert. 2006. Tetracycline residues in honey after hive treatment. Food Addit. Contam. 23: 265-273.

Menapace, D.M., and P. Hale. 1981 Citral and a combination of sodium propionate and potassium sorbate did not control chalkbrood. Am. Bee J. 121: 889-891.

Milne, C.P. 1983. Honey bee (*Hymenoptera: Apidae*) hygienic behavior and resistance to chalkbrood. Ann. Entomol. Soc. Am. 76: 384-387.

Moritz, R.F.A. 1988. A reevaluation of the two-locus model for hygienic behavior in honeybees (*Apis mellifera* L.). J. Hered. 79: 257-262.

Mutinelli, F. 2003. Practical application of antibacterial drugs for the control of honey bee diseases. Apiacta 38: 149-155.

Nelson, D.L., and T.A. Gochnauer. 1982. Field and laboratory studies on chalkbrood disease of honeybees. Am. Bee J. 122: 29–34.

O'Connor, E.B., Ross, R.P., and C. Hill. 2007. Applications of bacteriocins in the food industry, pp. 153-180. In M.A. Riley and O. Gillor [eds.], Research and applications in bacteriocins. Horizon Bioscience, Norfolk, United Kingdom.

Peng ,Y.S.C., Mussen, E., Fong, A., Montague, M.A., and T. Tyler. 1992. Effects of chlortetracycline of honey bee worker larvae reared in vitro. J. Invertebr. Pathol. 60: 127-133.

Puerta, F., Flores, J.M., Bustos, M., Padilla, P., and F. Campano. 1994. Chalkbrood development in honeybee brood under controlled conditions. Apidologie 25: 540-546.

Rothenbuhler, W.C. 1964. Behaviour genetics of nest cleaning honeybees. IV. Responses of F1 and backcross generations to disease killed brood. Am. Zool. 4: 111-123.

SAS Institute Inc. 2009. JMP®8 statistics and graphics guide, 2nd Ed. SAS Institute Inc, Cary, NC.

Schmuck, R. 2004. Effects of a chronic dietary exposure of the honeybee *Apis mellifera* (Hymenoptera: Apidae) to imidacloprid. Arch. Environ. Contam. Toxicol. 47: 471–478.

Spiltoir, C.F., and L.S. Olive. 1955. A reclassification of the genus *Pericystis* Betts. Mycologia 47: 238-244.

Spivak, M., and M. Gilliam. 1993. Facultative expression of hygienic behavior of honeybees in relation to disease resistance. J. Apic. Res. 32: 147-157.

Spivak, M., and G. S. Reuter. 1998. Performance of hygienic honey bee colonies in a commercial apiary. Apidologie 29: 291-302.

Suchail, S., Guez, D., and L.P. Belzunces. 2001. Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera*. Environ. Toxicol. Chem. 20: 2482–2486.

Thomas, L.V., and J. Delves-Broughton. 2005. Nisin, pp. 237-274. In P. M. Davidson, J. N. Sofos, & A. L. Branen (Eds.), Antimicrobials in food, 3rd ed. Taylor and Francis, Boca Raton, FL.

Thompson, T.S., Pernal, S.F., Noot, D.K., Melathopoulos, A.P., and J.P. van den Heever. 2007. Degradation of incurred tylosin to desmycosin-implications for residue analysis of honey. Anal. Chim. Acta 586: 304-311.

Toumanoff, C. 1951. Les maladies des abeilles [Diseases of bees]. Rev. Fr. Apic. Num. Spec. 68: 325.

Yakobson, B.A., Elad, D., Rosenthal, K., Kmer, I., Slovecky, I., and H. Efrat, 1991. A recent chalkbrood outbreak in Israel: attempts at therapeutic intervention, Am. Bee J. 131: 786.

Zaghloul, O.A, Mourad, A.K., El Kady, M.B., Nemat, F.M., and M.E. Morsy. 2005. Assessment of losses in honey yield due to the chalkbrood disease, with reference to the determination of its economic injury levels in Egypt. Commun. Agric. Appl. Biol. Sci. 70: 703-714.

Chapter 3: The Effects of Chalkbrood Disease on Honey Bee Colonies in Northern Alberta and the Efficacy of Lysozyme-HCl in the Suppression of Chalkbrood Disease in the Field

Introduction

Chalkbrood, a fungal disease of honey bee brood caused by *Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir (Spiltoir and Olive 1955) occurs worldwide (Ellis and Munn 2005). Identified by the hardened, chalk-like, black or white larval cadavers it produces, chalkbrood epizootics are variable and unpredictable (Befus-Nogel et al. 1992) but generally increase in incidence in the 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 have 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 in North America. 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; Martel et. al. 2006).

Lysozyme-HCl is an inexpensive, food-grade antimicrobial enzyme used in cheese and winemaking processes in the European Union, the 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). In *in vitro* larval rearing assays (Chapter 2), it has been shown that lysozyme-HCl is active against the fungous *A. apis*, the causative agent of chalkbrood disease in honey bees, although the mode of action is unknown.

Safe for both honey bee and human consumption (Johnson and Larson 2005; Chapter 2), 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 the subsequent economic impact of chalkbrood on beekeeping operations 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 anywhere from 1-37% of honey yields (Heath 1982; Yakobson et al. 1991) and up to 49% of foraging capacity (Heath 1982). Recent 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 was evaluated and the impacts of chalkbrood disease on: (*i*) colony population, (*ii*) honey production, and (*iii*) winter survival in newly established package colonies was assessed.

Methods

Colony Establishment and Management

Forty colonies were established April 24, 2007 at the AAFC Beaverlodge Research Farm (55° 18' N; 119° 17' W). One-kg "package bees" with queens (mixed race) were imported from New Zealand and hived onto irradiated (10 kGy, Iotron Industries Canada Inc, Port Coquitlam, BC) single brood chambers (nine frames, full depth Langstroth). 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 (Figure 3.1). Bottom boards were modified by removing the rim along the rear of the colonies in order to accommodate the removal of corrugated plastic sheets (Tenplast® 37 ×46 cm) that lay flat on the bottom of the

brood chamber. These sheets facilitated the counting of chalkbrood mummies removed from the comb by bees (Figure 3.2). Colonies were managed as single brood chambers throughout the experiment. Prior to or during the experiment, colonies were re-queened with New Zealand queens as needed.

At the time of establishment all colonies were treated with Fumagilin-B® (DIN 02231180, Medivet Pharmaceuticals High River, AB, Canada) in sucrose syrup according to label directions for package colonies (100 mg a.i./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 prior to start of experiment on 11 May 2007 using a Plexiglas™ grid (2.5 cm x 2.5 cm squares) and grouped into three strength categories based on adult bee population size (Figure 3.3). Colonies were randomly selected from within each of the three 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% (w/w) irradiated pollen, 35% (w/w)

commercial table grade sucrose, 5% (w/w) Brewer's yeast, 20% (w/w) sterile distilled water] containing chalkbrood mummies. Mummies were collected from diseased apiaries in the Beaverlodge area and homogenized in five 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 were used; each pollen contained approximately 1.56×10^7 spores. Control colonies received a similar pollen patty without the addition of homogenized mummies. Pollen patties were placed in the center of the colony on the top bars. 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% (w/v) 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, three and 10 days post inoculation and plated on PDY as previously described to confirm viability of *A. apis* spores.

Colony Treatment

Lysozyme-HCl (inovapure 300™, Neova Technologies, Abbotsford, B.C., Canada) was mixed in 1:1 (w/v) sucrose syrup prepared with table grade sucrose and applied to colonies using frame feeders. Inoculated colonies were untreated or given weekly applications of 600, 3000 or 6000 mg lysozyme-HCl for three weeks. The first treatments for all doses were dissolved in two liters of syrup,

while in subsequent treatments only one liter of syrup was used. The dates of application were 15, 22, and 29 May 2007 (Day 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 3 x 600 mg of lysozyme-HCl); **medium** (inoculated and treated with 3 x 3000 mg of lysozyme-HCl); **high** (inoculated and treated with 3 x 6000 mg of lysozyme-HCl).

Colony Assessments

Disease Severity and Bee Mortality

Black and white chalkbrood mummies and dead adult bees were collected and counted from (*i*) dead bee traps and (*ii*) 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 three days (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 by using a Plexiglas™ grid (2.5 cm x 2.5 cm squares). 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 (Figure 3.3). 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 seven days after the first treatment application and then weekly for three additional weeks. A

sample from each colony was also collected in the same manner three days prior to the start of the experiment before treatments were applied. Samples were stored at 5°C in 1.5 mL microfuge tubes until analyzed by Neova Technologies (Abbotsford, B.C., Canada) who employed an enzyme-linked immunobsorbent assay (ELISA) to determine lysozyme-HCl concentrations.

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 placing wrapping them in sterilized cheesecloth, dipping in 75% ethanol and immediately transferring for 60 seconds 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% chloremphenicol (10 g yeast, 10 g dextrose, 13.5 g KH_2PO_4, 10 g soluble starch, 20 g agar in 1 L 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. Following 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 the numbers of black and white chalkbrood mummies in uncapped cells were counted and colony population (unsealed brood, sealed brood, adult bees) was estimated.

Statistical Analysis

Colonies were excluded from the experiment when chronic queenlessness or diseases other than chalkbrood resulted in lack of brood any time prior to 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); 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 (Tukey-Kramer HSD; $\alpha = 0.05$).

Winter survival between treatment groups was compared using a chi-square 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, North Carolina).

Results

Disease Severity and Adult Bee Mortality

The mean number of total chalkbrood mummies collected daily from the traps and bottom boards (Figure 3.4) 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 13.7 and 12.5 mummies/d respectively, 16-19 fold more than the uninoculated and high treatment groups and four fold 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 (10.5/d) than the low treatment group (7.8/d) but significantly higher numbers of white mummies were collected (F = 87.75511; df = 4, 3526; $P <$ 0.0001) from the low treatment colonies (4.7/d) than the inoculated treatment group (3.1/d). Both the low and inoculated treatment groups produced significantly more black and white mummies than the uninoculated, medium and high treatment groups (Figure 3.4). 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 (Figure 3.5). On average, 34 to 37 mummies/week were counted in the inoculated and low treatment brood frames compared to the 3 to 8 mummies/week in the brood frames of 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 ($F_{\text{Frame Black Mummies}} = 12.34$; df = 4, 489; $P < 0.0001$, $F_{\text{Frame White Mummies}} = 9.63$; df = 4, 489; $P < 0.0001$). The numbers of white mummies in the inoculated and low treatment groups averaged 17.8 to 20.3/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 1.5-2.9 black mummies and 1.2-5.0 white mummies per week.

The numerical differences of the number of black mummies visible in the brood frames compared with the number of white mummies was not significant among treatment groups ($F = 0.35$; df = 4, 489; $P = 0.84$) or for the entire experiment ($F = 0.7615$; df = 1, 986; $P = 0.3831$) but the differences in the number of black mummies compared to the number of white mummies collected daily from the traps and bottom boards were significant both for the entire experiment ($F = 88.3566$; df = 1, 7060; $P < 0.0001$) and among treatment groups (Figure 3.6, 3.7). In the daily counts significantly more black (7.4/d) than white mummies were collected (Figure 3.6) from the inoculated colonies compared to the other

treatment groups (F = 65.85, df = 4, 3526; $P < 0.0001$). The low dose treatment group produced, on average three fold more black than white mummies on a daily basis and was significantly greater than the uninoculated, medium and high dose treatment groups (0.06-1.06/d), which were statistically similar.

A closer examination of the non transformed daily average of total mummies collected in each treatment group over the period of 14 May – 28 August 28 2007 reveals some general trends (Figure 3.8). Five days post inoculation chalkbrood mummies appeared in the traps and bottom boards of all inoculated treatment groups and peaked in number nine days after inoculation. Mummy count decreased almost immediately and 12 days post inoculation 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 days post inoculation 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 weeks post inoculation, 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 five weeks post inoculation 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 non significant

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; $P < 0.0406$). Post-hoc 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 (Figure 3.9). 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 over 4000 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; 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 (Figure 3.10). Repeated measure analysis of adult bee mortality over time (14 May – 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 following 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 two weeks from the start of the experiment until 10 July 2007 showed a significant effect of time for all three measures of the population (F_{Adult} = 160.27; df = 3.68, 102.91; $P < 0.0001$, F_{Sealed} = 56.78; df = 3.87, 108.31; $P < 0.0001$, $F_{Unsealed}$ = 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 (Figures 3.11, 3.12, 3.13). There was no significant treatment effect (F_{Adult} = 0.4732; df = 4,28; P = 0.7550, F_{Sealed} = 0.0350; df = 4,28; P = 0.9975, $F_{Unsealed}$ = 0.2296; df = 4,28; P = 0.9195) or time by treatment interaction for adult bee, sealed brood, or unsealed brood numbers (F_{Adult} = 0.7467; df = 14.70, 102.91; P = 0.7292, F_{Sealed} = 0.8458; df = 15.47, 108.31; P = 0.6283, $F_{Unsealed}$ = 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 (Figure 3.14). 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 (Figure 3.15).

Stability and Persistence of Lysozyme-HCl

Lysozyme-HCl activity was detected in all treatment groups given lysozyme-HCl (Figure 3.16) 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). Post-hoc comparisons revealed a significant difference in the effect of treatment over time when the inoculated high dose treatment group was contrasted with both of the inoculated 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 (Table 3.1).

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.

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; $P = 0.6849$) (Figure 3.17). 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 (Figure 3.18). 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 which averaged 0.5 to 1.8 chalkbrood mummies per colony.

Discussion

Lysozyme-HCl in the medium (3 x 3000 mg) and highest (3 x 6000 mg) doses tested was capable of suppressing mummy production in artificially inoculated colonies to levels similar to that of uninoculated untreated colonies (Figures 3.4,

3.5, 3.6, 3.7). Mean daily mummy production was five times lower in the inoculated medium dose treatment group and 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 five days post inoculation and a sharp decrease one week later, it was only the highest dose treatment group that suppressed mummy production until the end of the summer (Figure 3.7). Long-term suppression in the high dose treatment group may be the result of the significantly higher levels of lysozyme-HCl built up in the stored food cells surrounding the brood comb (Figure 3.16). The lowest dose (3 x 600 mg) 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 prior to inoculation and three chalkbrood mummies were collected from one colony prior to inoculation, indicating a pre-existing chalkbrood infection (Table 3.1, Figure 3.9). 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 pre-inoculation 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 (Figure 3.7). 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 (Figure 3.6). The colour variation in chalkbrood mummies is due to 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 behaviour is a heritable colony-level trait important in disease resistance (Rothenbuhler 1964) and studies have shown that colonies that exhibit this behaviour are more resistant to chalkbrood (Gilliam et al. 1983; Spivak and Reuter 1998). Hygienic behaviour 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 odours is higher in honey bees bred for hygienic behaviour than in bees from non-hygienic 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* (pre sporulation) was capable of inducing hygienic behaviour 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 non-sporulated larvae or if they have a greater quantity of the volatile phenethyl acetate. The colonies in this experiment were not assayed for hygienic behaviour 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.

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 (Figure 3.14) 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 health 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 $60-80 increase per colony at current honey values (USDA 2010) especially when the cost of the highest treatment administered (3 x 6000 mg 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 (Figure 3.15). 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 labour 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 1000-2000 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 2548 mummies over the entire experiment yielded 38 kg more honey than a colony that produced only 84 chalkbrood mummies (Figure 3.15).

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 (1999-2008) Alberta has produced 38% of Canadian honey yields and the Peace region alone produced 11% of total Canadian honey production (Statistics Canada 2000-2009). The mean honey yield per colony in the Peace region during that time period was 66 kg/colony, 7 kg more than the overall Albertan average and 5 kg more than the average Canadian yield (Alberta Agriculture and Food 2006; Alberta Agriculture and Rural Development 2008). 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 (Figure 3.11, 3.12, 3.13). The buildup of adult bee populations peaked 26 June (~ 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 (15-20 000 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 day post establishment to reach maximum population levels of 45-50 000 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 colonies 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 (Figure 3.18).

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 three days post inoculation and complete removal by three weeks in chalkbrood-susceptible colonies. Although there was a decrease in the number of mummies collected in this experiment two weeks post inoculation, 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 moulds, 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 pre-existing *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 in Beaverlodge, Alberta 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 (3 x 6000 mg) 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.

Figure 3.1. Dead bee traps (modified from Illies et al. 2002) attached to the front of experimental colonies shown from the front and the side. The mesh prevented the worker bees from removing dead adult bees and chalkbrood mummies from the colony, as they are unable to fly through with the cadavers. Instead, the cadavers were dropped in the drawer at the bottom of the trap.

Figure 3.2. Removable sheets facilitated the counting of chalkbrood mummies removed from the comb by bees. The rear bottom edge of the bottom board was removed and corrugated plastic sheets (Tenplast® 37 ×46 cm) were inserted.

Figure 3.3. Colony populations were assessed using a Plexiglas™ grid with 2.5 x 2.5 cm squares. Adult bees, sealed brood, and unsealed brood were measured. In this image, the area of adult bees on one side of a brood frame is being determined.

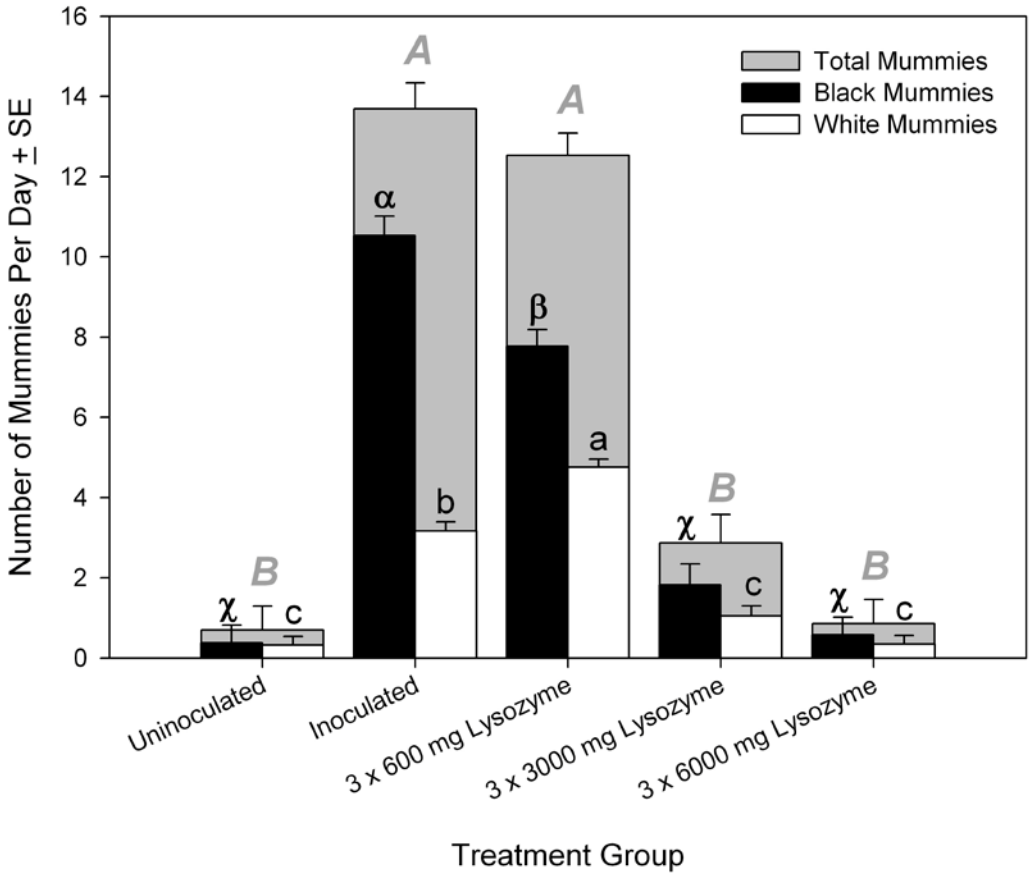

Figure 3.4. The mean daily number of mummies (total, black, white) collected on weekdays from traps and bottom boards for each treatment group from 14 May to 28 August 2007; collections on Monday were averaged over Saturday, Sunday and Monday. The numbers of total, black and white mummies collected were 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 (grey, uppercase, italicized letters, *A*); black mummy production (black, lowercase, greek letters, α); white mummy production (black, lowercase letters, a).

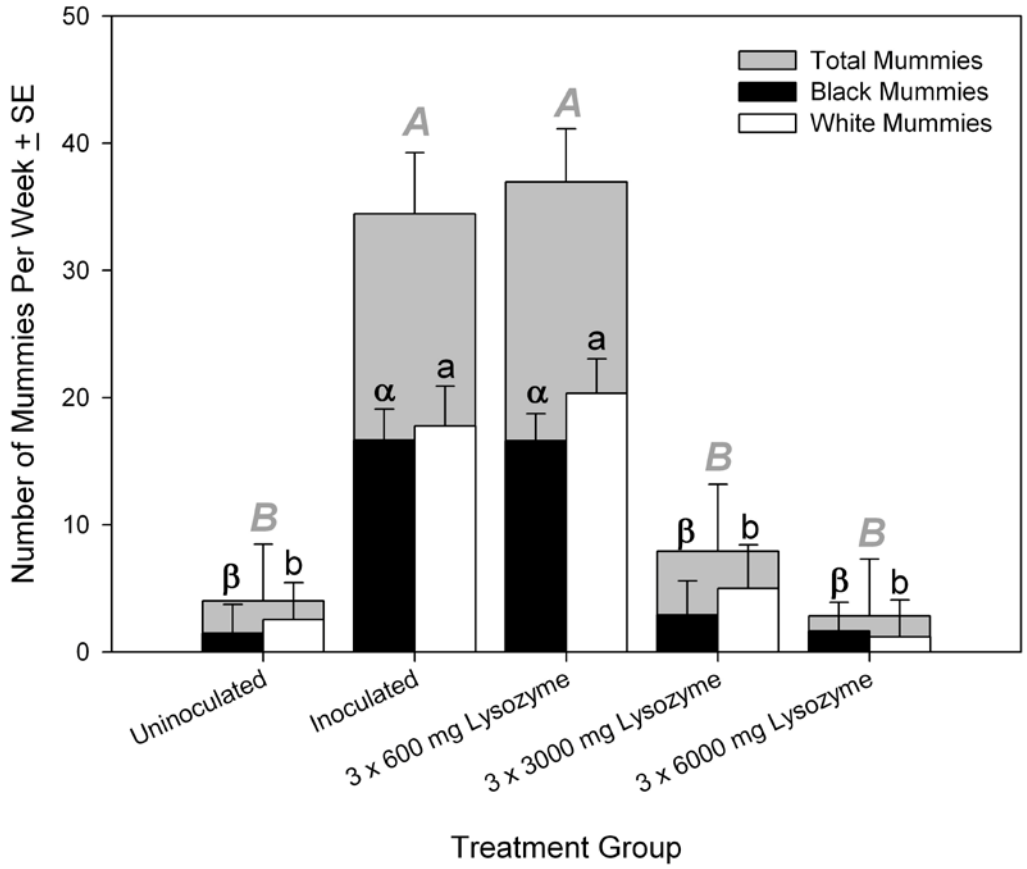

Figure 3.5. The mean number of visible chalkbrood mummies (black and white) in brood frames counted weekly for each treatment group from 14 May to 28 August 2007. The numbers of total, black and white mummies counted were analyzed separately. Treatments with different letters are significantly different at $\alpha = 0.05$ (Tukey-Kramer HSD). For each category different letter styles were used to indicate significance: total mummy production (grey, uppercase, italicized letters, *A*); black mummy production (black, lowercase, greek letters, α); white mummy production (black, lowercase letters, a).

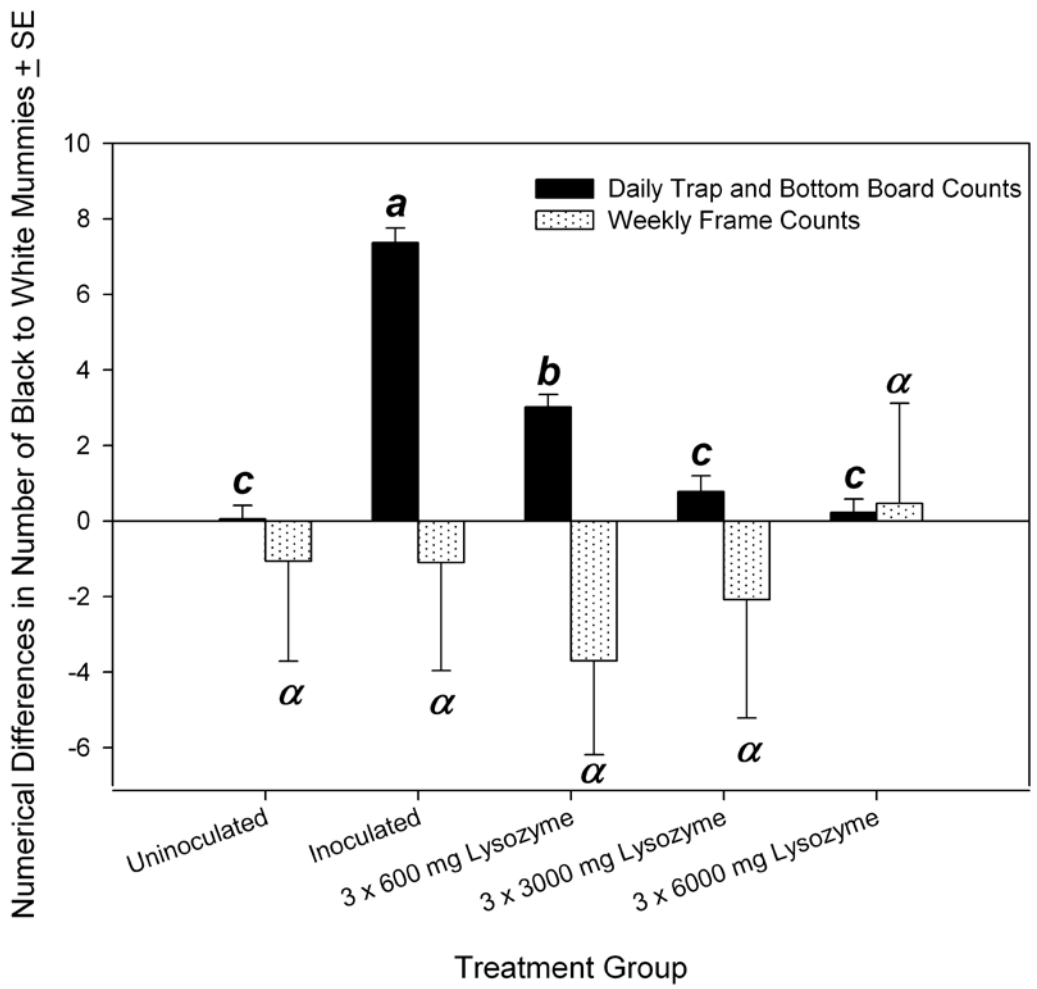

Figure 3.6. The mean numerical difference in number of black mummies compared with white mummies collected daily from traps and bottom boards or counted weekly in brood frames. Daily and weekly counts were analyzed separately. Treatments with different letters are significantly different at $\alpha = 0.05$ (Tukey-Kramer HSD). For each category different letter styles were used to indicate significance: daily counts (black, lowercase letters, a); weekly counts (black, lowercase, greek letters, α).

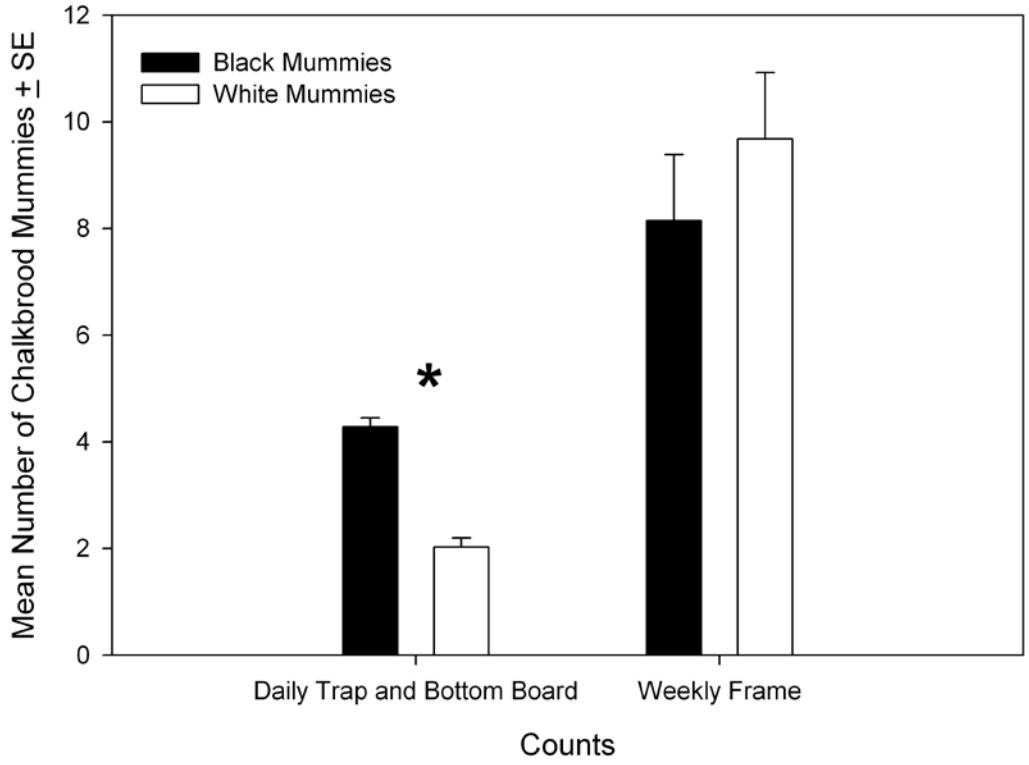

Figure 3.7. The mean number of black and white chalkbrood mummies collected daily from the traps and bottom boards or counted weekly in brood frames for all colonies in the experiment (n=33) from 14 May to 28 August 2007. Daily and weekly counts were analyzed separately. An asterisk (*) denotes a significant difference ($\alpha = 0.05$) in the number of black mummies compared to the number of white mummies counted.

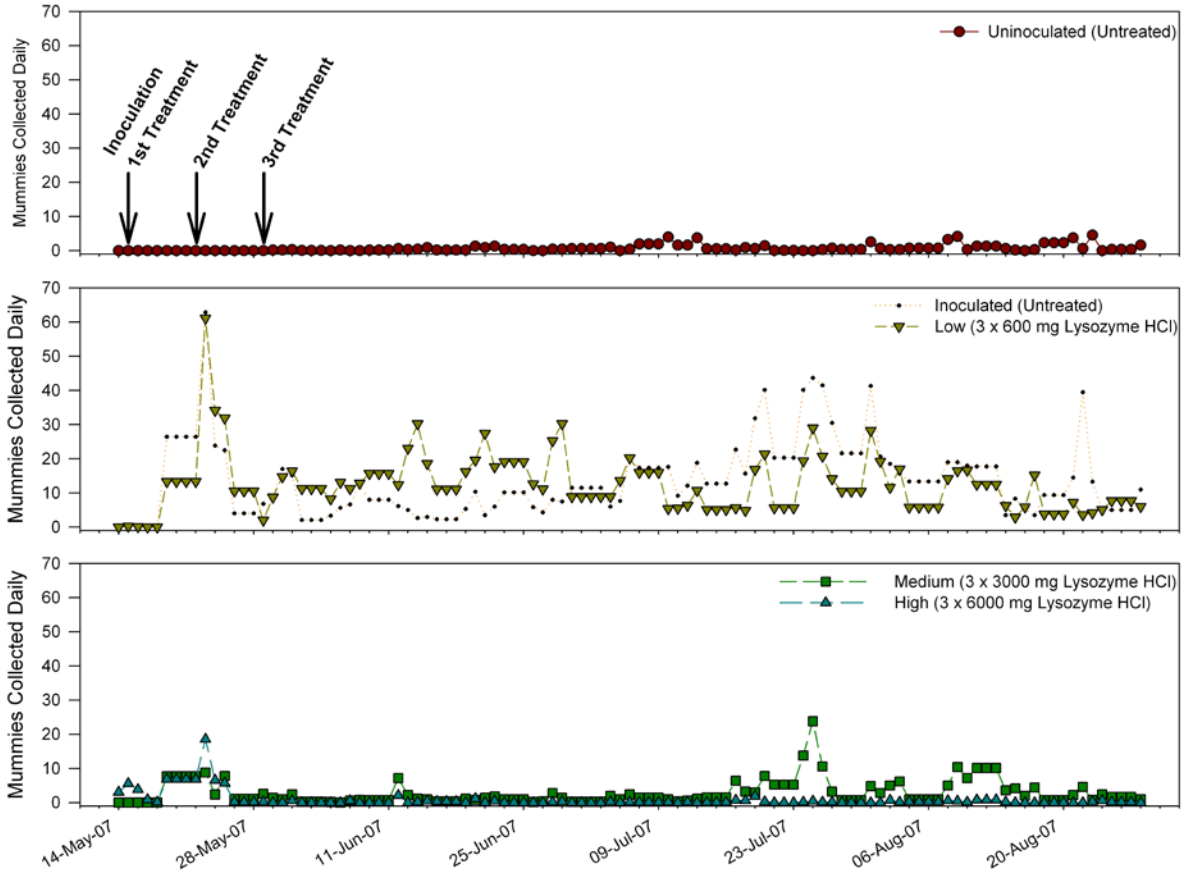

Figure 3.8. Mean number of chalkbrood mummies collected daily from traps and bottom boards for each treatment group from 14 May – 28 August 2007. Pollen patties (113 g) containing homogenized chalkbrood mummies were applied to all inoculated treatment groups (Inoculated, Low, Medium, High) and clean 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% (w/v) sucrose syrup containing 0 (Uninoculated, Inoculated), 600 (Low), 3000 (Medium), or 6000 mg (High) of lysozyme-HCl.

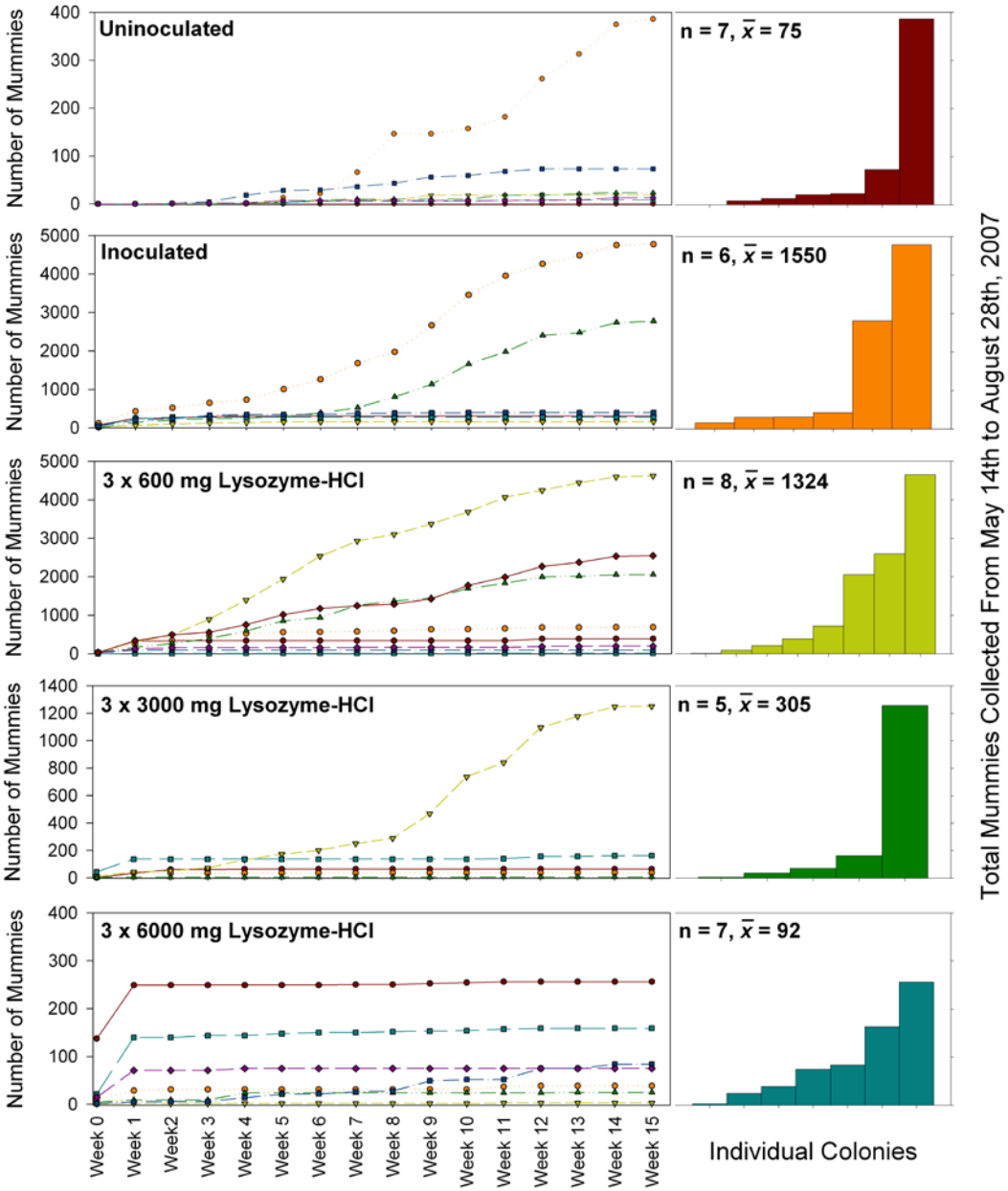

Figure 3.9. The weekly cumulative number of chalkbrood mummies collected daily from traps and bottom boards for individual colonies within each treatment group. The bar graphs show the total number of chalkbrood mummies collected from each colony from 14 May – 28 August 2007 for each treatment group. **Note: the scale of the y-axis is not the same for each treatment group.**

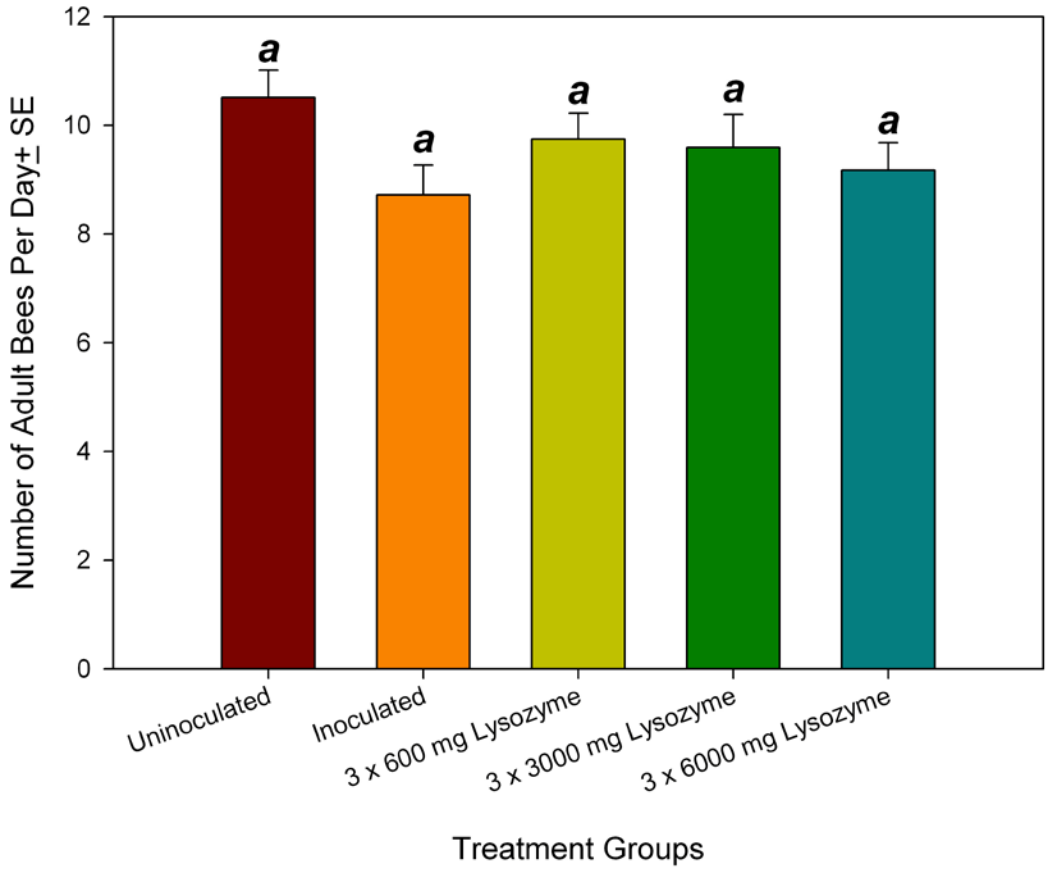

Figure 3.10. The average number of dead adult bees collected daily from traps and bottom boards compared among treatment groups from 14 May – 28 August 2007. Treatments were not significantly different at α =0.05 (Tukey-Kramer HSD).

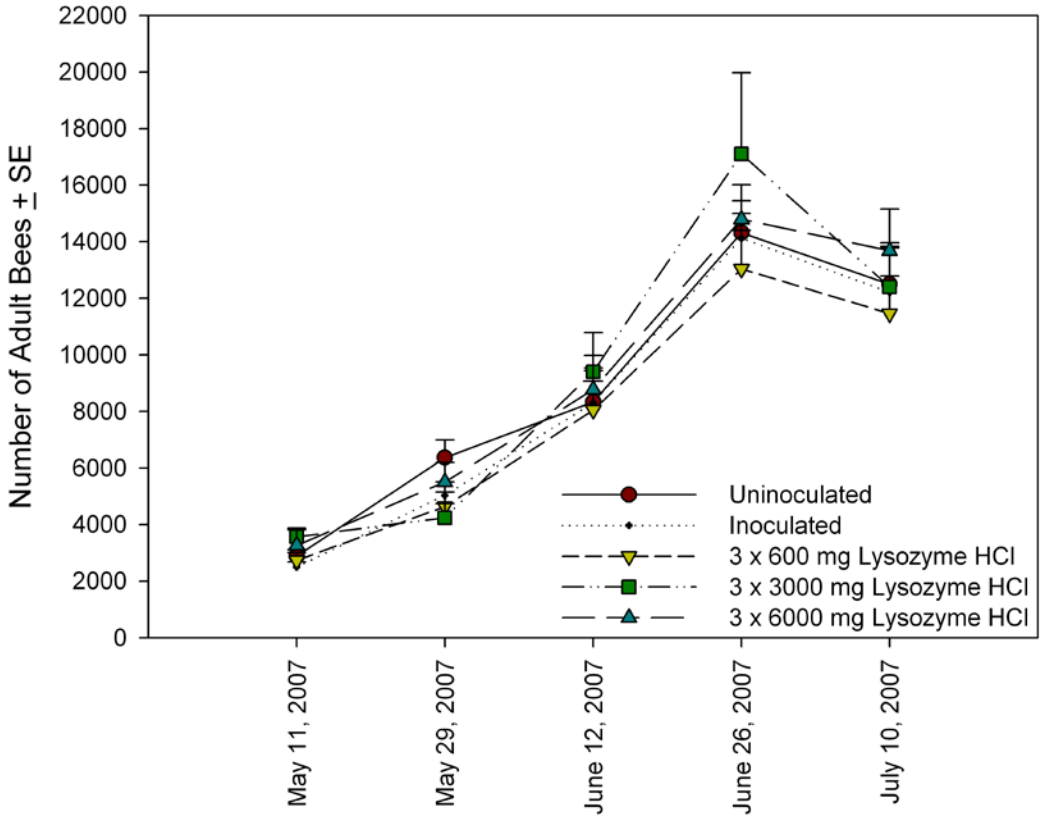

Figure 3.11. Mean number of adult bees per colony for each treatment group estimated on 11 May and then every 2 weeks from 29 May until 10 July 2007.

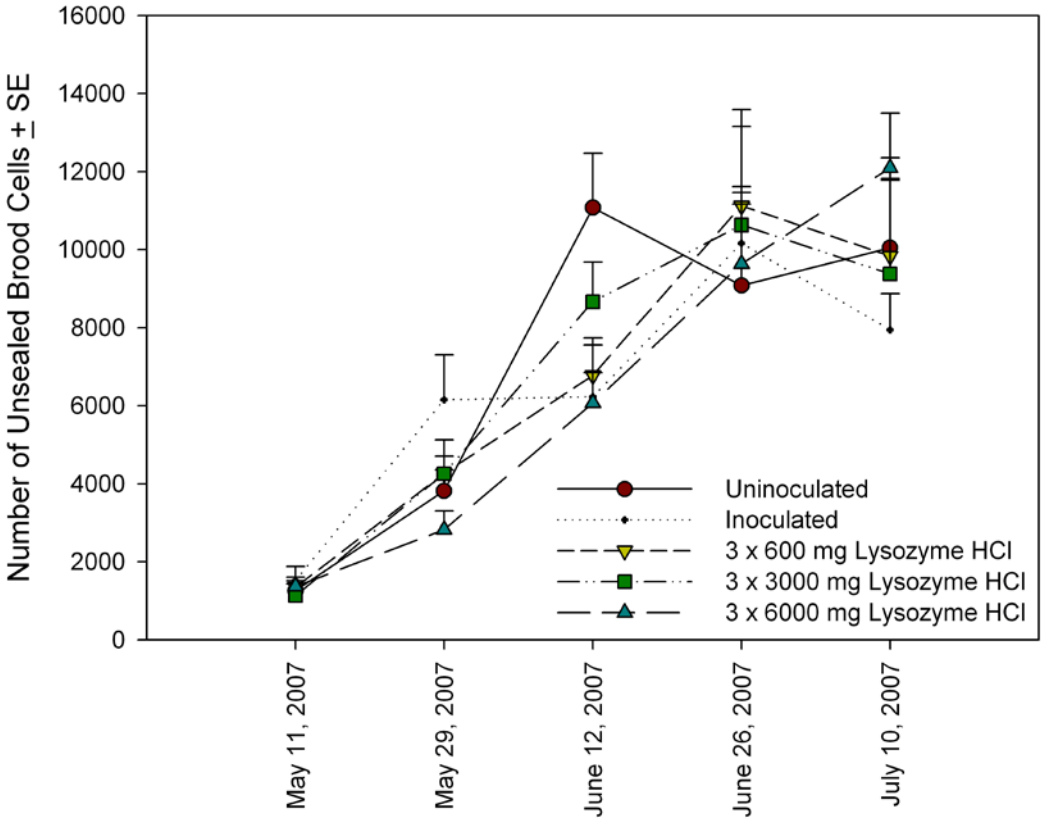

Figure 3.12. Mean number of sealed brood cells per colony for each treatment group estimated on 11 May and then every 2 weeks from 29 May until 10 July 2007.

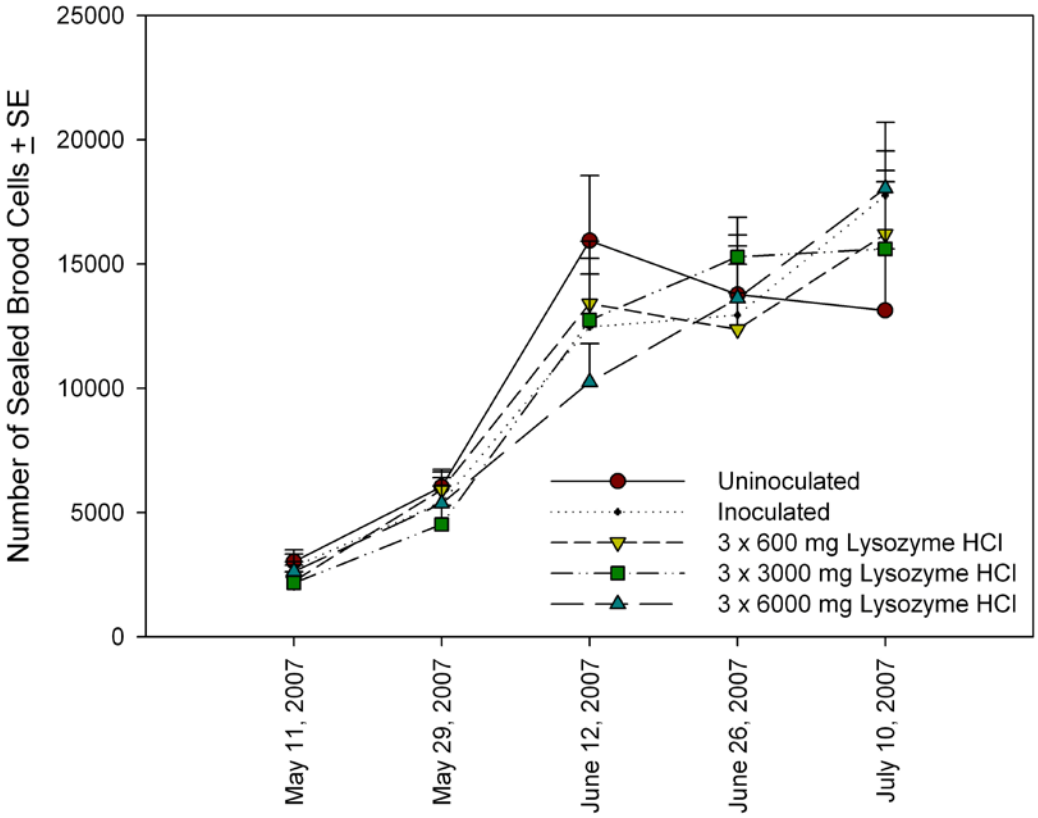

Figure 3.13. Mean number of unsealed brood cells per colony for each treatment group estimated on 11 May and then every 2 weeks from 29 May until 10 July 2007.

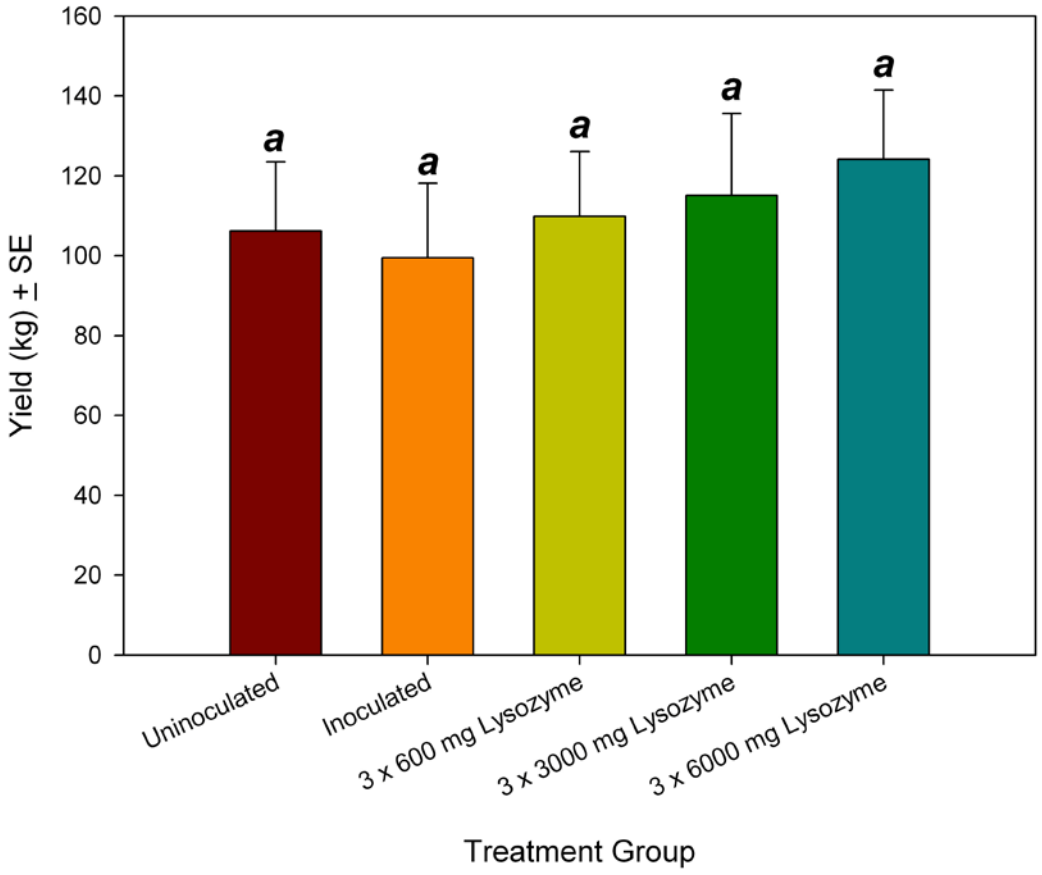

Figure 3.14. Mean honey production (kg) per colony compared among treatment groups. Honey was collected for the duration of the trial (14 May – 28 August 2007). Treatments were not significantly different at $\alpha = 0.05$ (Tukey-Kramer HSD).

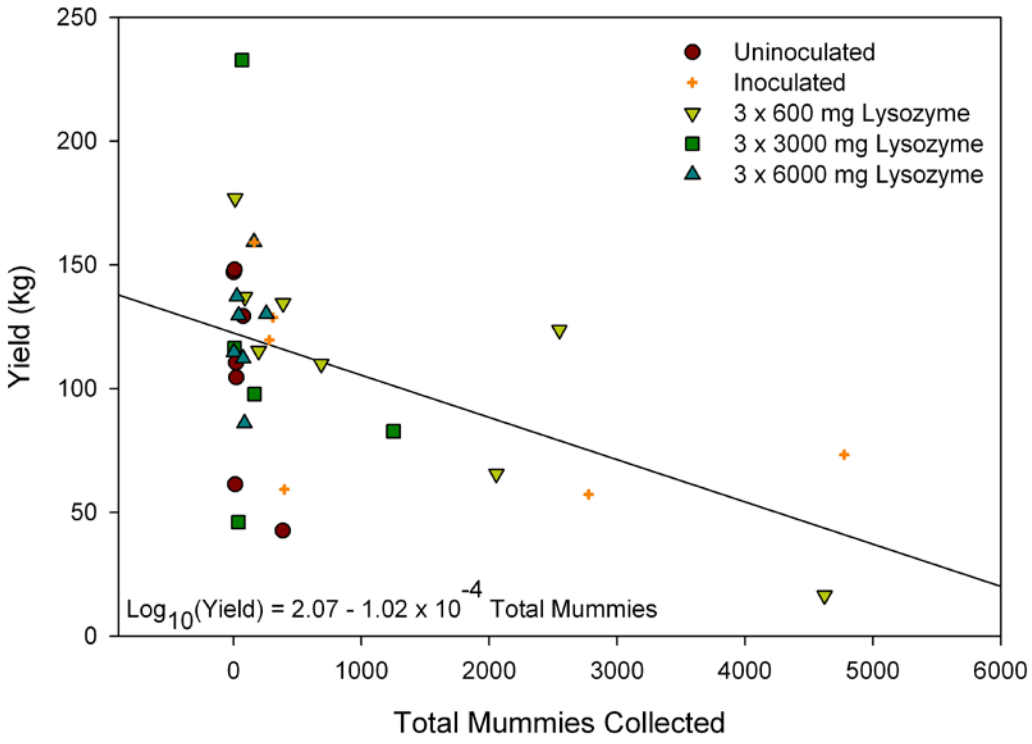

Figure 3.15. The relationship between total mummies collected daily from traps and bottom boards and honey yield (kg). Each data point represents an individual colony (n=33). Data presented in the figure is untransformed.

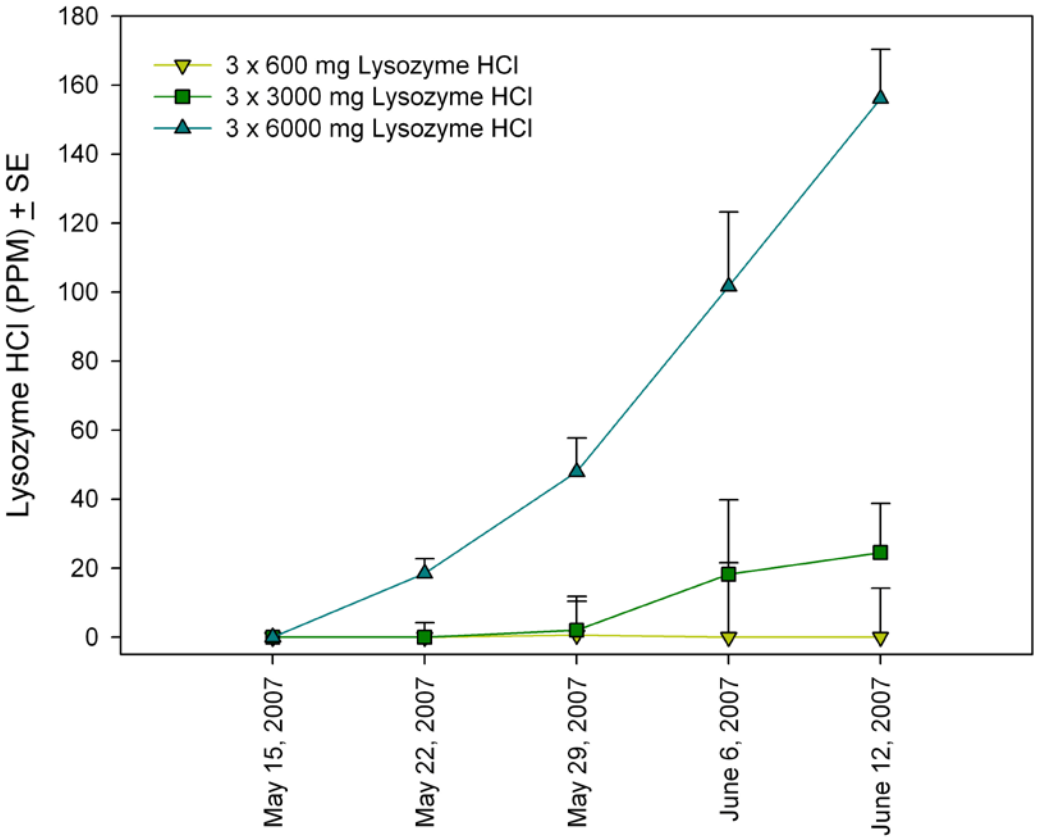

Figure 3.16. The mean amount (ppm) of lysozyme-HCl detected in the stored food collected from the outer edges of three brood frames of each colony compared among treatment groups that were administered three dosages of lysozyme-HCl. Colonies were sampled before the first treatment was applied and then weekly for four weeks afterwards.

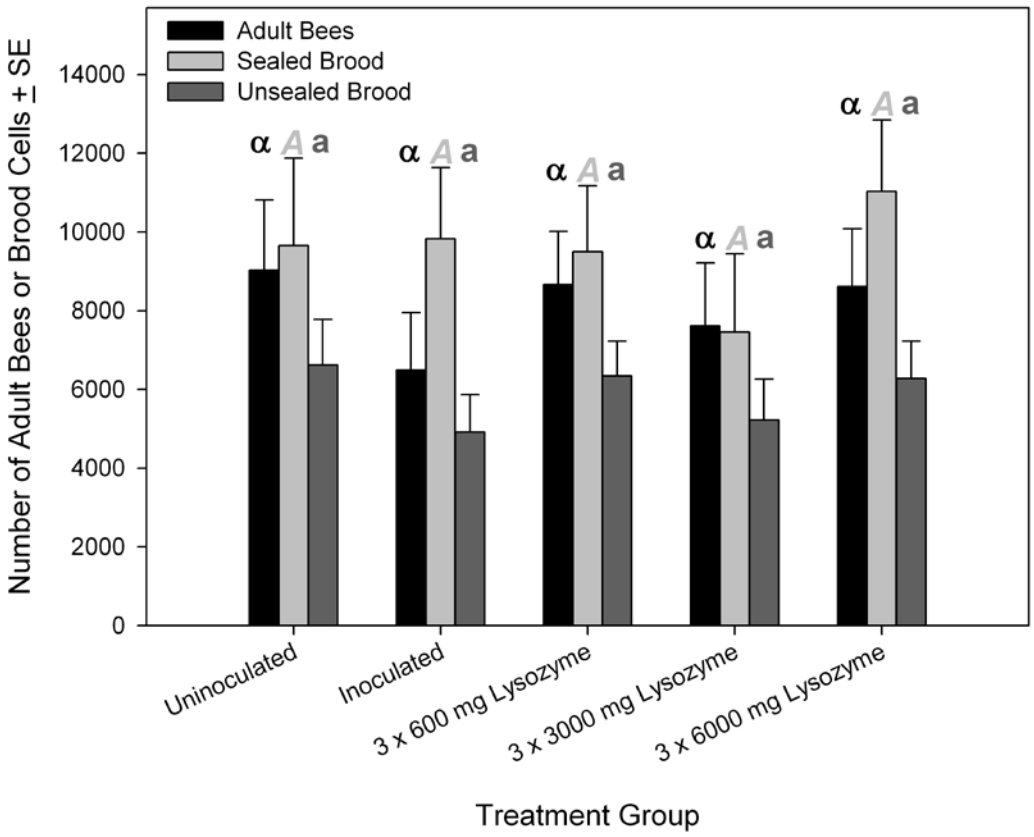

Figure 3.17. The average number of adult bees, sealed brood cells, and unsealed brood cells counted on 14 May 2008 for each treatment group. The number of adult bees, sealed brood cells, and unsealed brood cells were analyzed separately. Treatments with different letters are significantly different at $\alpha = 0.05$ (Tukey-Kramer HSD). For each category different letter styles were used to indicate significance: sealed brood cells (grey, uppercase, italicized letters, *A*); adult bee numbers (black, lowercase, greek letters, α); unsealed brood cells (black, lowercase letters, a).

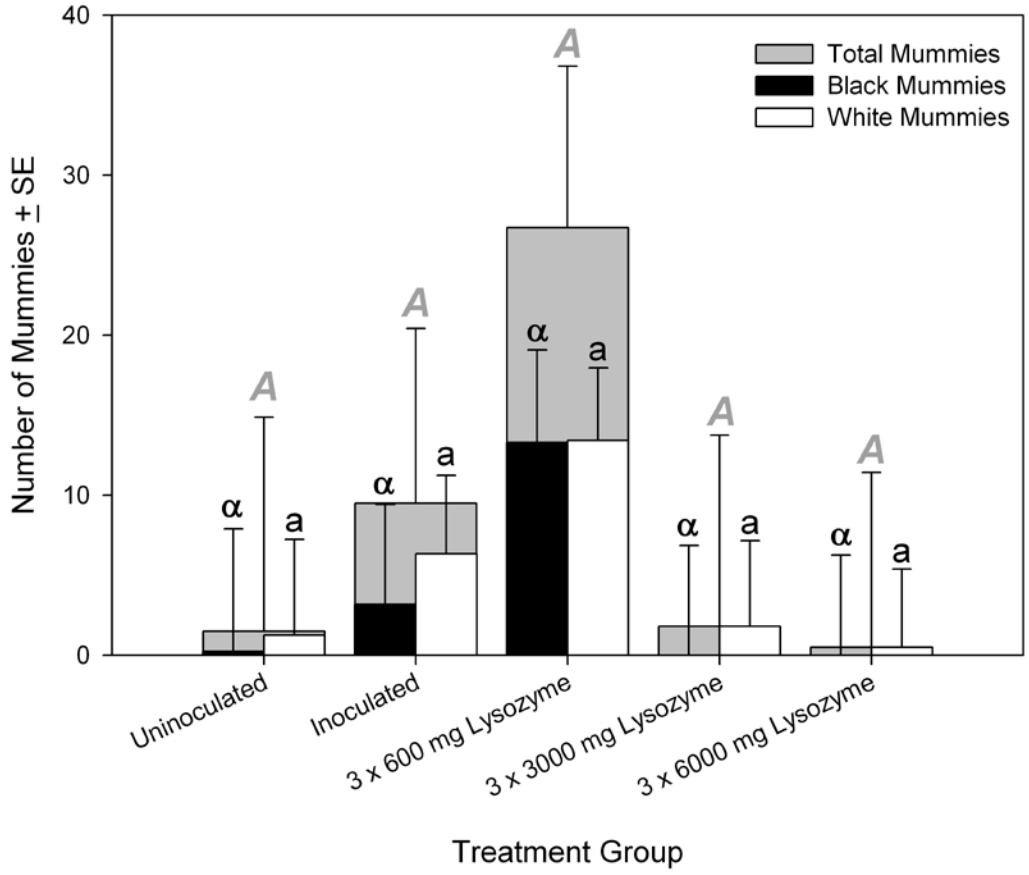

Figure 3.18. The average number of visible chalkbrood mummies (black and white) in brood frames counted on 14 May 2008 for each treatment group. Total, black and white mummies from each treatment group were analyzed separately. Treatments with different letters are significantly different at $\alpha = 0.05$ (Tukey-Kramer HSD). For each category different letter styles were used to indicate significance: total mummy production (grey, uppercase, italicized letters, *A*); black mummy production (black, lowercase, greek letters, α); white mummy production (black, lowercase letters, a). for total (*A*), black (α) and white (a) mummy counts.

Table 3.1. Percentage of samples (adult bee digestive tracts and larvae) from each treatment group collected weekly that were identified as chalkbrood positive. Three samples of each type at each sample date were cultured according to the methods of Anderson et al. (1997) and identity of *A. apis* confirmed microscopically.

Treatment	Sample	May 15	May 22	May 29	June 5	June 12
Uninoculated Untreated	adult	8.33	0.00	16.67	16.67	12.50
	larva	4.17	4.17	4.17	28.57	0.00
Inoculated Untreated	adult	0.00	16.67	20.83	12.50	0.00
	larva	4.17	4.17	8.33	37.50	14.29
Inoculated 3 x 600 mg Lysozyme HCl	adult	12.50	16.67	8.33	8.33	20.83
	larva	8.33	12.50	16.67	29.17	4.17
Inoculated 3 x 3000 mg Lysozyme HCl	adult	12.50	12.50	8.33	25.00	16.67
	larva	12.50	20.83	16.67	29.17	0.00
Inoculated 3 x 6000 mg Lysozyme HCl	adult	8.33	4.17	12.50	8.33	8.33
	larva	0.00	0.00	12.50	16.67	4.17

% of samples positive for chalkbrood (n=3 for each sample type at each date)

Literature Cited

Alberta Agriculture and Food. 2006. Alberta 2006 beekeepers' survey results. Alberta Agriculture and Food, Edmonton, AB, Canada. http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/sdd11876/$file/beekeepersreport.pdf?OpenElement

Alberta Agriculture and Rural Development. 2008. Alberta 2008 beekeepers' survey results. Alberta Agriculture and Rural Development, Edmonton, AB, Canada. http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/sdd12940/$file/report2008.pdf?OpenElement

Anderson, D.L., Giacon, H., and N. Gibson. 1997. Detection and thermal destruction of the chalkbrood fungus (*Ascosphaera apis*) in honey. J. Apic. Res. 36: 163-168.

Aronstein, K.A., and K.D. Murray. 2010. Chalkbrood disease in honey bees. J. Invertebr. Pathol. 103: S20-S29.

Befus-Nogel, J., Nelson, D.L., and L.P. Lefkovitch. 1992. Observations on the effect of management procedures on chalkbrood levels in honey bee (*Apis mellifera* L; Hymenoptera: Apidae) colonies. Bee Sci 2:20-24.

Berger, L.R., and R.S. Weiser. 1957. The β-glucosaminidase activity of egg-white lysozyme. Biochim. Biophys. Acta. 26: 517-521.

Bodenheimer, F.S. 1937. Studies in animal population. II. Seasonal population-trends of the honey-bee. Quart. Rev. Biol. 12: 406-425.

Davis, C., and W. Ward. 2003. Control of chalkbrood disease with natural products. Publ. No. 03/107, Rural Industries Research and Development Corporation, Kingston ACT, Australia.

Düring, K., Porsch, P., Mahn, A., Brinkmann, O., and W. Gieffers. 1999. The non-enzymatic microbicidal activity of lysozymes. FEBS Lett. 449: 93-100.

Ellis, J.D., and P.A. Munn. 2005. The worldwide health status of honey bees. Bee World 86: 88-101.

Gilliam, M. 1986. Infectivity and survival of the chalkbrood pathogen, *Ascosphaera apis*, in colonies of honey bees, *Apis mellifera*. Apidologie 17: 93-100.

Gilliam, M., and B.J. Lorenz. 1993. Enzymatic activity of strains of *Ascosphaera apis*, an entomopathogenic fungus of the honey bee, *Apis mellifera*. Apidologie 24: 19-23.

Gilliam, M., Taber, S.III., and G.V. Richardson. 1983. Hygienic behavior of honey bees in relation to chalkbrood disease. Apidologie 14: 29-39.

Gilliam, M., Taber, S.III., Lorenz, B.J., and D.B. Prest. 1988. Factors affecting development of chalkbrood disease in colonies of honey bees, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis*. J. Invertebr. Pathol. 52: 314-325.

Gramacho, K.P., and M. Spivak. 2003. Differences in olfactory sensitivity and behavioral responses among honey bees bred for hygienic behavior. Behav. Ecol. Sociobiol. 54: 472-479.

Griffin, D.H. 1994. Fungal physiology, 2nd ed. Wiley-Liss, New York, NY.

Guzmán-Novoa, E., and N.E. Gary. 1993. Genotypic variability of components of foraging behavior in honey bees (Hymenoptera: Apidae). J. Econ. Entomol. 86: 715-721.

Harbo, J.R. 1986. Effect of population size on brood production, worker survival and honey gain in colonies of honeybees. J. Apic. Res. 25: 22-29.

Heath, L.A.F. 1982. Development of chalk brood in a honey bee colony: a review. Bee World 63: 119-130.

Hornitzky, M. 2001. Literature review of chalkbrood–a fungal disease of honeybees. Publ. No. 01/150, Rural Industries Research and Development Corporation, Kingston ACT, Australia.

Ileana, N. 2007. Researches regarding the chalkbrood disease (*Ascosphaera apis*) of the honey bees. Bulletin USAMV-CN 64: 207-210.

Illies, I., Mühlen, W., Dücker, G., and N. Sachser. 2002. The influence of different bee traps on undertaking behaviour of the honey bee (*Apis mellifera*) and development of a new trap. Apidologie 33: 315-326.

Jākobsons, B. 2005. Biological treatment of chalkbrood in honey bees. Ph.D. dissertation, Latvia University of Agriculture, Jelgava, Latvia.

Johnson, E.A., and A.E. Larson. 2005. Lysozyme, pp. 361–387. In P. M. Davidson, J. N. Sofos, & A. L. Branen (Eds.), Antimicrobials in food, 3rd ed. Taylor and Francis, Boca Raton, FL.

Johnson, R.N., Zaman, M.T., Decelle, M.M. Siegel, A.J., Tarpy, D.R., Siegel E.C., and P.T. Starks. 2005. Multiple micro-organisms in chalkbrood mummies: evidence and implications. J. Apic. Res. 44: 29-32.

Lapidge, K.L., Oldroyd, B.P., and M. Spivak. 2002. Seven suggestive quantitative trait loci influence hygienic behavior of honey bees. Naturwissenschaften 89: 565-568.

Martel, A.C., Zegganea, S., Drajnudela, P., Faucona, J.P., and M. Auber. 2006. Tetracycline residues in honey after hive treatment. Food Addit. Contam. A 23: 265-273.

Masterman, R., Ross, R., Mesce, K., and M. Spivak. 2001. Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis mellifera* L). J. Comp. Physiol., A 187: 441–452.

McKee, B. 2003. Prevention of residues in honey: A future perspective. Apiacta, 38: 173-177.

Menapace, D., and P. Hale. 1981. Citral and a combination of sodium propionate and potassium sorbate did not control chalkbrood. Am. Bee J. 121: 889-891.

Nelson, D.L., and T.A. Gochnauer. 1982. Field and laboratory studies on chalkbrood disease of honeybees. Am. Bee J. 122: 29–34.

Nelson D.L., and S.C. Jay. 1972. Population growth and honey yield studies of package bee colonies in Manitoba. II. Colonies initiated with four package sizes on one date. Man. Entomol. 6: 17-22.

Nelson D.L., and S.C. Jay. 1982. Producing honey in the Canadian prairies using package bees. Bee World 63: 110-117.

Oxley, P.R., Spivak, M., and B. Oldroyd. 2010. Six quantitative trait loci influence task thresholds for hygienic behaviour in honeybees (*Apis mellifera*). Mol. Ecol. 19: 1452-1461.

Pankiw, P. 1968. The influence of environmental conditions on brood rearing, build-up, and honey production of package bee colonies. Can. Entomol. 100: 127-134.

Rothenbuhler, W.C. 1964. Behaviour genetics of nest cleaning honeybees. IV. Responses of F_1 and backcross generations to disease killed brood. Am. Zool. 4: 111-123.

Seeley, T.D. 1989. The honey bee colony as a superorganism. Am. Sci. 77: 546-553.

Shimanuki H., and D.A. Knox. 2000. Diagnosis of honey bee diseases. US Department of Agriculture, Agriculture Handbook No. AH-690.

Spiltoir, C.F., and L.S. Olive. 1955. A reclassification of the genus *Pericystis* Betts. Mycologia 47: 238-244.

Spivak, M., and G. S. Reuter. 1998. Performance of hygienic honey bee colonies in a commercial apiary. Apidologie 29: 291-302.

Statistics Canada. 2000-2005. Production and value of honey and maple products. Catalogue 23–221-XIB, Ottawa, Canada.

Statistics Canada. 2006. Production and value of honey and maple products. Catalogue 23–221-XIE, Ottawa, Canada.

Statistics Canada. 2007-2009. Production and value of honey and maple products. Catalogue 23–221-X, Ottawa, Canada.

Strominger J.L., and D.J. Tipper. 1974. Structure of bacterial cell walls: the lysozyme substrate, pp. 169–184. In: E.F. Osserman., R.E. Canfield and S. Beychok (eds), Lysozyme. Academic Press, New York, NY.

Swanson, J.A.I., Torto, B., Kells, S.A., Mesce, K.A., Tumlinson, J.H., and M. Spivak. 2009. Odorants that induce hygienic behavior in honeybees: identification of volatile compounds in chalkbrood-infected honeybee larvae. J. Chem. Ecol. 35: 1108–1116.

Taber S.III. 1986. Breeding bees resistant to chalkbrood. Am. Bee J. 126: 823-825.

Taber, S.III., Sackett, R., and J. Mills. 1975. A possible control for chalkbrood disease. Amer. Bee J. 115: 20.

U.S. Department of Agriculture. 2010. National Honey Report March 17, 2010. USDA, Beltsville, MD. http://search.ams.usda.gov/mndms/2010/03/FV20100317Mhoney.pdf

Westcott, L.C., and M.L. Winston. 1999. Chemical acaricides in *Apis mellifera* (Hymenoptera: Apidae) colonies; do they cause nonlethal effects? Can. Entomol. 131: 363-371.

Winston, M.L. 1987. The biology of the honey bee, Harvard University Press, Cambridge, Mass.

Yakobson, B.A., Elad, D., Rosenthal, K., Kamer, I., Slovecky, I. and H. Efrat. 1991. A recent chalkbrood outbreak in Israel: attempts at therapeutic intervention, Am. Bee J. 131: 786.

Zaghloul, O.A, Mourad, A.K., El Kady, M.B., Nemat, F.M., and M.E. Morsy. 2005. Assessment of losses in honey yield due to the chalkbrood disease, with reference to the determination of its economic injury levels in Egypt. Commun. Agric. Appl. Biol. Sci. 70: 703-714.

Chapter 4: Synthesis and Future Research

Research Summary

Chalkbrood, a fungal disease of honey bee larvae, is caused by the spore-forming heterothallic fungus *Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir (Spiltoir and Olive 1955) and occurs worldwide (Ellis and Munn 2005). Identified by the hardened chalk-like black and white larval cadavers it produces, chalkbrood epizootics are variable and unpredictable (Befus-Nogel et al. 1992). 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 chalkbrood is rarely lethal to a colony, it is an economically important disease and losses attributed to chalkbrood have been estimated to vary from 1-37% of honey yields (Heath 1982, Yakobsen et al. 1991; Zaghoul et al. 2005). Despite numerous studies of possible chemical treatments (Hornitzky 2001) currently there is no registered chemotherapeutic treatment available for chalkbrood disease in North America. The aim of this thesis was to evaluate two food-safe natural compounds, lysozyme-HCl and nisin, for use in honey bee colonies as a control for chalkbrood and to assess the impacts of chalkbrood disease on honey bee colonies.

To establish the potential of lysozyme-HCl and nisin as safe effective treatments for chalkbrood disease in the colony, the acute and chronic oral toxicity of both compounds on adult worker bees at the age typically associated with nursing or larval care (7-9 d) was assessed. Oral toxicity tests are normally carried out at room temperature, but as the compounds evaluated were meant to be administered

in the colony at hive temperatures, toxicity was evaluated at 34 °C (EPPO 1992). Nisin was not acutely toxic at the highest dose tested, 6400 µg a.i /bee but caused 50% mortality in 14 days at the lowest concentration fed continuously to the bees (1.6 µg/mL). The mortality may not have been due to nisin but rather the extremely high levels of sodium chloride in the commercial preparation used (Novasin™). Based on the chronic toxicity results, nisin was eliminated as a potential treatment for chalkbrood disease at the colony level. Lysozyme-HCl (inovapure™ 300) was also not acutely toxic at the highest dose tested, 6400 µg a.i./bee. At 21 d, mortality was not significantly different between bees continuously fed 1.6 µg/mL lysozyme-HCl and bees consuming sucrose syrup. The low toxicity, acutely and chronically, of lysozyme-HCl to adult nurse bees indicated it could be a safe treatment to apply at the colony level.

To evaluate the efficacy of lysozyme-HCl on chalkbrood disease in honey bee larvae outside of the colony environment, the *in vitro* larval rearing assay established by Peng et al. (1992) for the study of *Paenibacillus larvae* (sensu Genersch et al. 2006), the causative agent of American foulbrood was modified. To enable consistent infection of worker larvae with chalkbrood disease, the larvae were inoculated with *A. apis* spores (1.0×10^8 spores/mL diet), the causative agent of chalkbrood disease, 72 hours after the initial transfer as larvae 3-4 d old are most susceptible to infection (Bailey 1967). At the time of transfer to the pupation trays, larvae were removed from the incubator and chilled to 28-30°C for 1 h to increase infection and mummification (Bailey 1967, Puerta et al.

1994) and daily larval transfer was eliminated to reduce the labour involved in the assay. The inoculum for the *in vitro* larval rearing assay was prepared from black chalkbrood mummies homogenized in sterile water but it has been shown that chalkbrood mummies are not pure sources of *A. apis* spores and contain a variety of yeasts, moulds and bacteria (Johnson et al. 2005). However, it is unlikely that under natural conditions in the colony that larvae would encounter uncontaminated *A. apis* spores.

The assay was successful and mortality attributed to chalkbrood disease in untreated inoculated larvae was 89±2.8% and 1.0±2.8% in untreated uninoculated larvae. Lysozyme-HCl, tested against inoculated larvae was therapeutic at the lowest dose tested, 0.75% (7.5 μg/mL) and levels of adult emergence were similar to the uninoculated untreated control. However, mortality attributed to chalkbrood infection was similar (20-23%) at all doses of lysozyme-HCl tested (0.75, 1.5, 3.0%) indicating that lysozyme-HCl is highly fungistatic but not fungicidal. Additionally, pupal mortality was increased at the highest dose evaluated (3.0%) suggesting that lysozyme-HCl may be toxic to larvae at that dose. The results of the *in vitro* rearing assay demonstrated that lysozyme-HCl was capable of suppressing chalkbrood disease in larvae outside of the colony environment.

As laboratory experiments established that lysozyme-HCl was non-toxic and efficacious against chalkbrood disease in honey bee larvae, a field study was

conducted to determine a therapeutic dose of lysozyme-HCl at the colony level. A secondary goal of the field trial was to assess the impacts of chalkbrood disease on colony population, honey production, and winter survival in newly established package colonies as the economic impact of chalkbrood disease on colonies in northern Alberta are not well studied.

Colonies were successfully inoculated using pollen patties that contained homogenized chalkbrood mummies as per the methods described by Gilliam et al. (1988) and all inoculated colonies produced mummies within one week of inoculation. The first mummies in the uninoculated colonies appeared 15 days after the other colonies in the experiment were inoculated. Spores were detected in the all colonies pre-inoculation and the presence of mummies in the uninoculated colonies may have resulted from the pre-existing infection but spores may also have been transmitted from the inoculated colonies in the apiary as a result of drifting bees. Treatments of lysozyme-HCl were administered in sugar syrup as beekeepers routinely feed colonies in the spring and fall with syrup and if successful, the treatment could easily be integrated into established management practices. Lysozyme-HCl at the medium (3 x 3000 mg) and highest (3 x 6000 mg) doses tested was capable of suppressing mummy production in artificially inoculated colonies to levels similar to uninoculated colonies without any significant affects on adult bee mortality. Suppression of mummy production in the highest dose tested was season-long and most likely the result of increasing

quantities of lysozyme-HCl stored in the food surrounding the brood cells as the treatment was consumed.

Even though mummy production was significantly different, there were no significant differences in colony populations or winter survival among treatment groups. As chalkbrood disease increases in severity, increased larval death should translate into fewer adult worker bees and impact colony strength but the consequences of larval death may not be immediate or straightforward. The ability of the colony to respond to disturbance (Seeley 1989) may mean that the impact of chalkbrood disease is minimal unless severe and prolonged.

The most surprising result of this experiment was the discovery that black chalkbrood mummies were being removed preferentially. The numbers of white and black mummies visible in the frames were not significantly different but the numbers of black mummies that were removed by the bees and collected from the trap were significantly higher than the numbers of white mummies. One explanation for this finding is that volatiles emitted from chalkbrood mummies that elicit hygienic behavior (uncapping and removal of diseased larvae) in worker bees are present in higher concentrations in black mummies and once uncapped are immediately removed. It may be that white mummies are uncapped but not removed immediately.

The overall numbers of black and white mummies collected for the entire experiment was 68 and 32% respectively. In a previous field experiment conducted in Beaverlodge the ratios of black and white mummies collected were equal (Nelson and Gochnauer 1982). Gilliam et al. (1978, 1983) also reported similar results in laboratory and field trials but in a separate field experiment found the numbers of black mummies to be 65% of the total mummies collected (Gilliam 1986). The increased numbers of black mummies produced in this field trial may be the result of increased strain diversity as *A. apis* is heterothallic, requiring both a + and – strain to produce the fruiting bodies that give black chalkbrood mummies their colour. The colonies were artificially inoculated with chalkbrood mummies from the Beaverlodge area but *A. apis* spores were detected both in the larvae and adult bees prior to the start of the experiment indicating a pre-existing infection that was likely imported with the package bees from New Zealand. Interestingly, De Jong (1976 as cited in Heath 1985) conjectured that chalkbrood was first introduced to Canada in package bees imported from New Zealand in 1968. However, Aronstein and Murray (2010) have hypothesized that white mummies are not the result of colonization of a single unmated strain of *A. apis* but remain white as the fruiting bodies have not had the time or the right conditions to develop. It may be that in experiments where the conditions are ideal for spore development, more black chalkbrood mummies are produced.

The genetic background of the honey bee queens used in each colony was unknown. They were imported from the same source but that does not signify a

high level of relatedness and there is genetic variation in both honey production and chalkbrood susceptibility (Guzmán-Novoa and Gary 1993; Spivak and Gilliam 1993). There were high levels of variability in chalkbrood mummy production independent of the treatment effect. For example, within the inoculated treatment group, total mummy production in individual colonies ranged from 162 to 4784 mummies but it is not known if this was a result of different levels of hygienic behaviour. Given time, the colonies could have been assayed for hygienic behaviour to determine the variation in chalkbrood resistance present in the experimental population (Spivak and Gilliam 1993). However, resistance to chalkbrood brood disease is not confined to adult worker bee behaviours. Recently, it has been shown that larvae within colonies and among strains differ in susceptibility to infection (Jensen et al. 2009). Similar to American foulbrood, another common honey bee brood disease, there may be multiple heritable resistance mechanisms (Brødsgaard and Hansen 2003). Strains of *A. apis* strains also vary in virulence (Gliński 1982; Jākobsons 2005) and differences in disease severity may be the result of more virulent or novel strains.

It has been shown that colonies exhibiting high levels of hygienic behaviour produce significantly more honey than colonies that do not, but these traits may not be linked (Spivak and Reuter 1998). Honey production varied between colonies within the same treatment group independent of mummy production. Two colonies producing similar numbers of mummies (387 and 398) within the same treatment group had a difference in honey yield of 166 kg, which may be the

result of differences in worker foraging behaviours between colonies (Guzmán-Novoa and Gary 1993). However, chalkbrood production may not truly reflect infection levels in the colony as diseased larvae are often cannibalized (Riessberger-Gallé et al. 2001). In this experiment honey production was not significantly different between treatment groups but there was a significant negative relationship between the number of chalkbrood mummies a colony produced and honey yield. Interestingly, honey yield increased with increasing doses of lysozyme-HCl and the difference between the high dose treatment group and the untreated uninoculated and inoculated controls was 18-24 kg which is an increase of $60-80 per colony at current Canadian honey prices (USDA 2010).

Applications and Recommendations

Chalkbrood is considered a factorial disease requiring both the presence of spores in the larval gut and environmental stress before it is expressed (Flores et al. 2004). To understand chalkbrood disease in honey bee colonies, the disease triangle is a useful paradigm. Borrowed from plant pathology, the disease triangle is composed of three interacting elements: host, pathogen and environment (James 2008). Vectors of the pathogen can add a fourth dimension, which in the case of chalkbrood includes beekeepers. As this study has shown, each of these elements can be quite variable and can have a great impact on expression of chalkbrood disease in the colony (Figure 4.1). Successful management of chalkbrood disease should be based on all four components and not the use of therapeutic agents alone. Although beekeepers cannot control all aspects of the environment they

can choose apiary locations that are not cool and wet, as well as managing colonies for strength and food shortages. To reduce the spread of spores in their operation they can sanitize and disinfect contaminated equipment and ensure the bee stocks they are importing are disease-free. Lastly, they can use stock that has been selected for hygienic behavior or disease resistance.

The complete eradication of *A. apis* is not likely (Hornitzky 2001) and until we completely understand the pathology of the fungus, outbreaks will continue to be unpredictable. I have demonstrated that lysozyme-HCl is capable of suppressing chalkbrood disease in colonies when administered in three weekly treatments of 6000 mg in sugar syrup and at that dose is capable of controlling the disease for the entire summer season. Lysozyme-HCl is also having positive effect on honey yield that may not be the result of mummy suppression alone. The effects of lysozyme-HCl on other colony pathogens are not well characterized but there has been some research. Studies on the effects of lysozyme on *Nosema apis* Zander have been contradictory (Nagornaya et al. 2003; Maistrello et al. 2008) and in preliminary investigations, lysozyme-HCl was partially therapeutic in larvae infected with American foulbrood (Van Haga et al. unpublished).

Medication of honey bee colonies can be problematic as honey is a food product. Treatments applied to the colony that are not immediately consumed are stored as honey in the colony and may persist for extended periods of time (Argauer and Moats 1991; Thompson et al. 2007). Generally, treatments applied to the colony

have time restrictions to minimize residues in the extracted honey. Oxytetracycline-HCl, the antibiotic used for the treatment of American foulbrood in North America must not be applied during the main honey flow and treatment should cease four weeks before the main honey flow starts (Lafrenière and Ostermann 2010). As concerns over antibiotic residues in food products such as honey increase, investigations into the control of chalkbrood using natural or food grade products have been of great interest (Davis and Ward 2003). Because lysozyme-HCl has been granted GRAS (generally regarded as safe) status by the American Food and Drug Administration and accepted for use in food processing by the Joint FAO/WHO Expert Committee on Food Additives (Johnson and Larson 2005), residue concerns are not an issue and treatment time restrictions are unnecessary. However, approval for use of lysozyme-HCl on honey bee colonies for the treatment of chalkbrood disease in honey bee colonies must be granted by Health Canada before beekeepers can integrate it into their disease management strategies.

Future Research

Further investigations on the use of lysozyme-HCl on honey bee colonies should address the possibility of resistance. Although natural antimicrobials like lysozyme-HCl are non-selective inhibitors and generally do not target specific cell sites like antibiotics (Davidson and Harrison 2002), incidences of acquired resistance can occur. Development of resistance to lysozyme by various bacteria has been reported in laboratory experiments (Litwack and Prasad 1962; Gravesen et al. 2002) but resistance is often genetically unstable and bacteria revert to

susceptible levels when lysozyme is removed from the growth medium (Brumfitt 1959; Russel 1991). However, there has been evidence of stable acquired resistance to lysozyme by *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 in laboratory experiments (Özcengiz and Alaeddinoglu 1991). Consequently, the possibility of resistance to lysozyme-HCl when used a therapeutic agent for honey bee larval diseases should not be ignored.

Additionally, lysozyme-HCl may interact with treatments applied to colonies for other bee diseases; oxytetracycline-HCl, tylosin tartrate, Fumagilin-B®, and various acaricides are all applied to honey bee colonies. Lysozyme has been shown to work synergistically with a variety of antimycotics and antibiotics (Bukharin et al. 1986; Samaranayake et al. 2009) but drug combinations can be antagonistic (Hemaiswarya et al. 2008). The effects of lysozyme-HCl and Fumagilin-B® on honey bee colonies should be investigated as Fumagilin-B® is applied in sugar syrup and the potential for beekeepers to apply the treatments simultaneously exists.

It may also be of interest to study the economic impacts of chalkbrood in regions where the honey flow is extended and the production season is longer to understand the geographical implications of chalkbrood disease. Studies from both Israel and Egypt have demonstrated that chalkbrood is a serious economic disease in those regions (Yakobsen et al. 1991; Zaghoul et al. 2005) whereas in Romania, it is only of minor importance (Ileana 2007). Geographical differences

in management practices, climate, honey bee stocks and *A. apis* strains may all contribute to disease severity.

Our ability to understand and predict chalkbrood epizootics requires detailed knowledge of the pathogenesis of the disease (Genersch et al. 2010) and further study on the impacts of chalkbrood disease should control for host genetic variability that exists for chalkbrood susceptibility and honey production. Much attention has focused on the variability of the host but the effect of strain differences and strain competition on the development of chalkbrood in the colony and transmission between colonies should be investigated. Although this study has not utilized molecular methods, the entire genome of *A. apis* has been sequenced (Qin et al. 2006) and studies at the genomic and molecular level are already contributing to our understanding.

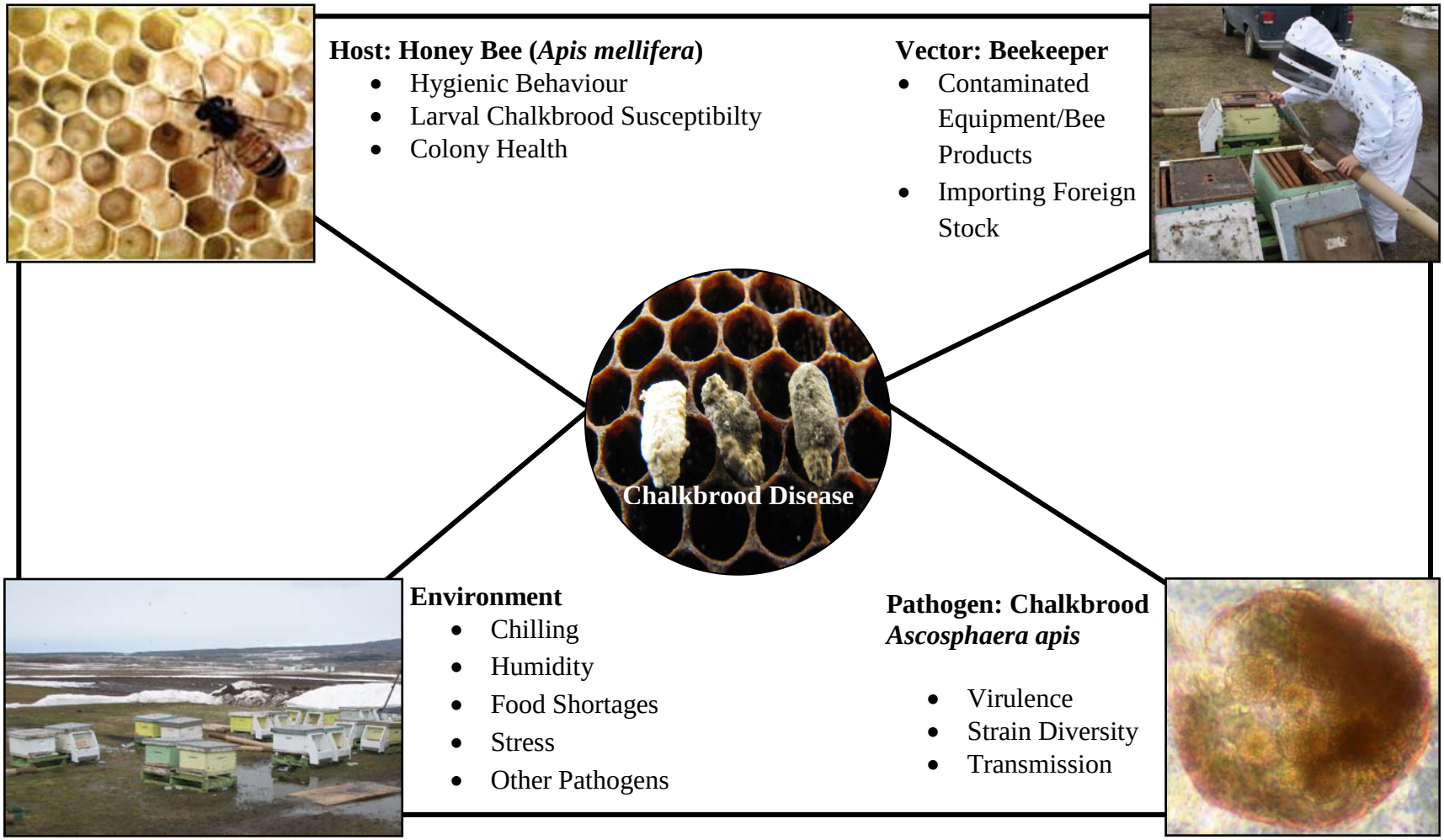

Figure 4.1. The disease pyramid for honey bees and chalkbrood disease (adapted from James 2008).

Literature Cited

Argauer, R.J., and W.A. Moats. 1991. Degradation of oxytetracycline in honey as measured by fluorescence and liquid chromatographic assays. Apidologie 22: 109–115.

Aronstein, K.A., and K.D. Murray. 2010. Chalkbrood disease in honey bees. J. Invertebr. Pathol. 103: S20-S29.

Bailey, L. 1967. The effect of temperature on the pathogenicity of the fungus *Ascosphaera apis* for larvae of the honeybee, *Apis mellifera*, pp. 162–167. In P.A. Van der Laan [ed], Insect pathology and microbial control. North Holland Publishing Co., Amsterdam, Netherlands.

Befus-Nogel, J., Nelson, D.L., and P. Lefkovich. 1992. Observations on the effect of management procedures on chalkbrood levels in honey bee (*Apis mellifera* L; Hymenoptera: Apidae) colonies. Bee Sci 2:20-24.

Brødsgaard, C.J., and H. Hansen. 2003. Tolerance mechanisms against American foulbrood in honey bee larvae and colonies. Apiacta 38: 114-124.

Brumfitt, W. 1959. The mechanism of development of resistance to lysozyme by some gram-positive bacteria and its results. Br. J. Exp. Pathol. 40: 441-451.

Bukharin, O.V., Zykova, L.S., and N.F. Tarasenko. 1986. Experimental and clinical study of the use of lysozyme in combination with chemotherapeutic agents. Antibiot Med Biotekhnol. 31: 917-920.

Davidson, P.M., and M.A. Harrison. 2002. Resistance and adaptation to food antimicrobials, sanitizers, and other process controls. Food Technol. 56: 60–78.

Davis, C., and W. Ward. 2003. Control of chalkbrood disease with natural products. Publ. No. 03/107, Rural Industries Research and Development Corporation, Kingston ACT, Australia.

De Jong, D. 1976. A study of chalk brood of honey bees. MSc. Thesis, Cornell University, Ithaca, USA.

Ellis, J.D., and P.A. Munn, 2005. The worldwide health status of honey bees. Bee World 86: 88-101.

European and Mediterranean Plant Protection Organization/Organisation européenne et méditerranéenne pour la protection des plantes. 1992. Guidelines on test methods for evaluating the side-effects of plant protection products on honeybees. Bull. OEPP 22: 203-215.

Flores, J.M., Gutierrez, I., and F. Puerta. 2004. Oxytetracycline as a predisposing condition for chalkbrood in honeybee. Vet. Microbiol. 103: 195-199.

Genersch, E., Forsgren, E., Pentikäinen, J., Ashiralieva, A., Rauch, S., Kilwinski, J., and I. Fries. 2006. Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. Int. J. Syst. Evol. Microbiol. 56: 501-511.

Gilliam, M. 1986. Infectivity and survival of the chalkbrood pathogen, *Ascosphaera apis*, in colonies of honey bees, *Apis mellifera*. Apidologie 17: 93-100.

Gilliam, M., Taber, S.III., and J.B. Rose. 1978. Chalkbrood disease of honey bees, *Apis mellifera* L.: a progress report. Apidologie 9: 75-89.

Gilliam, M., Taber, S.III., and G.V. Richardson. 1983. Hygienic behavior of honey bees in relation to chalkbrood disease. Apidologie 14: 29-39.

Gilliam, M., Taber, S.III, Lorenz B.J., and D.B. Prest. 1988. Factors affecting development of chalkbrood disease in colonies of honey bees, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis*, J. Invertebr. Pathol. 52: 314-325.

Gliński, Z. 1982. Studies on pathogenicity of *Ascosphaera apis* for larvae of the honeybee *Apis mellifera* L. Part II. Relationships between biochemical types and virulence of *A. apis*. Ann. Univ. Mariae Curie-Sklodowska, Sect. DD 37: 69-77.

Gravesen, A., Jydegaard Axelsen, A.M, Mendes da Silva, J., Hansen, T.B., and S. Knøchel. 2002. Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. Appl. Environ. Microbiol. 68: 756-764.

Guzmán-Novoa, E., and N.E. Gary. 1993. Genotypic variability of components of foraging behavior in honey bees (Hymenoptera: Apidae). J. Econ. Entomol. 86: 715-721.

Heath, L.A.F. 1982. Development of chalk brood in a honey bee colony: a review. Bee World 63: 119-130.

Heath, L.A.F. 1985. Occurrence and distribution of chalk brood disease of honeybees. Bee World 66: 9–15.

Hemaiswarya, S., Kruthiventi, A.K., and M. Doble. 2008. Synergism between natural products and antibiotics against infectious diseases. Phytomedicine 15: 639-652.

Hornitzky, M. 2001. Literature review of chalkbrood–a fungal disease of honeybees. Publ. No. 01/150, Rural Industries Research and Development Corporation, Kingston ACT, Australia.

Jākobsons, B. 2005. Biological treatment of chalkbrood in honey bees. Ph.D. dissertation, Latvia University of Agriculture, Jelgava, Latvia.

James, R.R. 2008. The problem of disease when domesticating bees, pp. 124-141. In R.R James and T.L. Pitts-Sanger (eds.), Bee pollination in agricultural ecosystems. Oxford University Press, New York, NY.

Jensen, A.B., Pedersen, B.V., and J. Eilenberg. 2009. Differential susceptibility across honey bee colonies in larval chalkbrood resistance. Apidologie 40: 524-534.

Johnson, E.A., and A.E. Larson. 2005. Lysozyme, pp. 361–387. In P. M. Davidson, J. N. Sofos, & A. L. Branen (Eds.), Antimicrobials in food,3rd ed. Taylor & Francis, Boca Raton, FL.

Johnson, R.N., Zaman, M.T., Decelle, M.M. Siegel, A.J., Tarpy, D.R., Siegel, E.C., and P.T. Starks. 2005. Multiple micro-organisms in chalkbrood mummies: evidence and implications. J. Apic. Res. 44: 29-32.

Lafrenière, R., and D. Ostermann. 2010. Recommendations for administering antibiotics and acaricides to honey bees. http://www.gov.mb.ca/agriculture////crops/honey/pdf/bha01s00.pdf.

Litwack, G., and A.L.N. Prasad. 1962. Development of lysozyme resistance in *Micrococcus lysodeikticus* and *Sarcina lutea*. Nature 196: 143-145.

Maistrello, L., Lodesani, M., Costa, C., Leonardi, F., Marani, G., Caldon, M., Mutinelli, F., and A. Granato. 2008. Screening of natural compounds for the control of nosema disease in honeybees (*Apis mellifera*). Apidologie 39: 436-445.

Nagornaya, I., Yefimenko, T.M., Omeltchenko, Y.P., and A.O. Arkhypov. 2003. Lysozyme and its role during honey bee nosematosis, pp.71. In Materialy z XL Naukowej Konferencji Pszczelarskiej, 11-12 March 2003, Pulawy, Poland.

Nelson, D.L., and T.A. Gochnauer. 1982. Field and laboratory studies on chalkbrood disease of honeybees. Am. Bee J. 122: 29–34.

Özcengiz G., and N.G. Alaeddinoglu. 1991. Bacilysin production and sporulation in *Bacillus subtilis*. Curr. Microbiol. 23: 61-64.

Peng C.Y.S., Mussen E., Fong A., Montigue M.A., and T. Tyler. 1992. Effects of chlortetracycline on honey bee worker larvae reared *in vitro*. J. Invertebr. Pathol. 60: 127-133.

Puerta, F., Flores, J.M., Bustos, M., Padilla, P., and F. Campano. 1994. Chalkbrood development in honeybee brood under controlled conditions. Apidologie 25: 540-546.

Riessberger-Gallé, U., Von Der Ohe, W., and K. Crailsheim. 2001. Adult honeybee's resistance against *Paenibacillus larvae* larvae, the causative agent of the American foulbrood. J. Invertebr. Pathol. 77: 231-236.

Russel, A.D. 1991. Mechanisms of bacterial resistance to non-antibiotics: food additives and food and pharmaceutical preservatives. J. Appl. Bacteriol. 71: 191-201.

Samaranayake, Y.H., Cheung, B.P.K., Parahitiyawa, N., Seneviratne, C.J., Yau, J.Y.Y., Yeung K.W.S., and L.P. Samaranayake. 2009. Synergistic activity of lysozyme and antifungal agents against *Candida albicans* biofilms on denture acrylic surfaces. Arch. Oral Biol. 54: 115-126.

Seeley, T.D. 1989. The honey bee colony as a superorganism. Am. Sci. 77: 546-553.

Spiltoir, C.F., and L.S. Olive. 1955. A reclassification of the genus *Pericystis* Betts. Mycologia 47: 238-244.

Spivak, M. and M. Gilliam. 1993. Facultative expression of hygienic behavior of honeybees in relation to disease resistance. J. Apic. Res. 32: 147-157.

Spivak, M., and G. S. Reuter. 1998. Performance of hygienic honey bee colonies in a commercial apiary. Apidologie 29: 291-302

Thompson, T.S., Pernal, S.F., Noot, D.K., Melathopoulos, A.P., and J.P. van den Heever. 2007. Degradation of incurred tylosin to desmycosin-implications for residue analysis of honey. Anal. Chim. Acta 586: 304-311.

U.S. Department of Agriculture. 2010. National Honey Report March 17, 2010. USDA, Beltsville, MD.
http://search.ams.usda.gov/mndms/2010/03/FV20100317Mhoney.pdf

Yakobson, B.A., Elad, D., Rosenthal, K., Kmer, I., Slovecky, I., and H. Efrat, 1991. A recent chalkbrood outbreak in Israel: attempts at therapeutic intervention, Am. Bee J. 131: 786.

Zaghloul, O.A, Mourad, A.K., El Kady, M.B., Nemat, F.M., and M.E. Morsy. 2005. Assessment of losses in honey yield due to the chalkbrood disease, with reference to the determination of its economic injury levels in Egypt. Commun. Agric. Appl. Biol. Sci. 70: 703-714.

Appendix 1: Chapter 3 Statistical Tests and Results

Description of Statistical Test	Results
Number of chalkbrood mummies collected daily from the traps and bottom boards compared among treatment groups (ANOVA)	**Total: F = 112.5413; df = 4, 3526; P <0.0001** **Black: F = 102.48; df = 4, 3526; P <0.0001** **White: F = 87.75511; df = 4, 3526; P <0.0001**
Number of chalkbrood mummies counted in the brood frames during the weekly colony inspections compared among treatment groups (ANOVA)	**Total: F = 14.2929; df = 4, 489; P <0.0001** **Black: F = 12.34; df = 4, 489; P <0.0001** **White: F = 9.63; df = 4, 489; P <0.0001**
Numerical differences in the number of black compared to white mummies counted in the brood frames during the weekly colony inspections (ANOVA)	Among Treatments: F = 0.35; df = 4, 489; P =0.84 All Colonies: F = 0.7615; df = 1, 986; P = 0.3831
Numerical differences in the number of black compared to white mummies collected daily from the traps and bottom boards (ANOVA)	**Among Treatments: F = 65.85; df = 4, 3526; P <0.0001** **All Colonies: F = 88.3566; df = 1, 7060; P <0.0001**
Repeated measures analysis of daily mummy counts (square root transformed) over the duration of the experiment (106 d) compared among treatment groups (MANOVA)	Treatment: F = 2.63; df = 4, 28; P = 0.0552 **Time: F = 6.43; df = 7.12, 199.43; P <0.0001** **Treatment*Time: F = 1.57; df = 28.48, 199.43; P < 0.0406** **Post-Hoc Comparison: F = 3.05; df = 7.12, 199.43; P = 0.0043**
Number of dead adult bees collected daily from traps and bottom boards compared among treatment groups (ANOVA)	F = 1.65; df = 4, 3526; P =0.1597
Repeated measure analysis of daily adult bee mortality over the duration of the experiment (106 d) compared among treatment groups (MANOVA)	Treatment: F = 0.5639; df = 4, 28; P =0.6908 **Time: F = 20.4678; df = 17.221, 482.2; P <0.0001** Treatment*Time: F = 0.8136; df =68.885, 482.2; P =0.8547
Repeated measures analysis of adult bee population (number of adult bees) counted 11 May and then every two weeks from 29 May to 10 July 2007 compared among treatment groups (MANOVA)	Treatment: F = 0.4732; df = 4, 28; P = 0.7550 **Time: F = 160.27; df = 3.68, 102.91; P < 0.0001** Treatment*Time F = 0.7467; df = 14.70, 102.91; P = 0.7292

Description of Statistical Test	**Results**
Repeated measures analysis of number of number of sealed brood cells counted 11 May and then every two weeks from 29 May to 10 July 2007 compared among treatment groups (MANOVA)	Treatment: F = 0.0350; df = 4, 28; P = 0.9975 **Time: F = 56.78; df = 3.87, 108.31; P < 0.0001** Treatment*Time: F = 0.8458; df = 15.47, 108.31; P = 0.6283
Repeated measures analysis of number of unsealed brood cells counted 11 May and then every two weeks from 29 May to 10 July 2007 compared among treatment groups (MANOVA)	Treatment: F = 0.2296; df = 4, 28; P = 0.9195 **Time: F = 35.89; df = 2.82, 78.87; P < 0.0001** Treatment*Time: F =1.10; df = 11.27, 78.87; P = 0.3738
Mean honey yield (kg) compared among treatment groups (ANOVA)	F = 0.2703; df = 4, 28; P = 0.8946
The relationship between total mummies collected from traps and bottom boards and honey yield in kg (log_{10} transformed) (Linear Regression)	**F = 16.03; df = 1,31; P =0.0004; R^2=0.34**
Repeated measures analysis of the amount of lysozyme-HCl detected in the stored food surrounding brood frames between 15 May and 12 June 2007 compared among treatment groups (MANOVA)	**Treatment: F = 45.88; df = 4,28; P < 0.0001** **Time: F = 5.81; df = 2.36,66; P = 0.003** **Treatment*Time: F = 3.63; df = 9.43,66; P = 0.0008** **Post-Hoc Comparison: F = 9.07; df = 2.36,66; P = 0.0002**
Percentage of colonies surviving the winter compared among treatment groups (Chi Square Test)	χ2=6.18; df = 4,33; P = 0.1502
Number of adult bees counted the following spring (14 May 2008) compared among treatment groups (ANOVA)	F = 0.4643; df = 4, 23; P = 0.7612
Number of sealed brood cells counted the following spring (14 May 2008) compared among treatment groups (ANOVA)	F = 0.4497; df = 4, 23; P = 0.7715
Number of unsealed brood cells counted the following spring (14 May 2008) compared among treatment groups (ANOVA)	F = 0.5731; df = 4, 23; P = 0.6849
Number of chalkbrood mummies counted in the brood frames the following spring (14 May 2008) compared among treatment groups (ANOVA)	Total: F = 1.088; df = 4, 23; P = 0.3857 Black: F = 0.8952; df = 4, 23; P = 0.4828 White: F = 1.279; df = 4,23; P = 0.3071